Genetic and Functional Characterisation of ALS-linked Genes TBK1, OPTN and MATR3

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Genetic and Functional Characterisation of ALS-linked Genes: *TBK1*, *OPTN* and *MATR3*

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Declaration

I hereby declare that, as the author of this thesis, the work reported here was performed by myself unless otherwise stated. Any collaborative effort and assistance provided by others have been acknowledged and all previous work referenced accordingly. This work has not been submitted for any other degree.

Abstract

Amyotrophic Lateral Sclerosis (ALS) is a degenerative disease predominantly affecting motor neurons in the brain and spinal cord. ALS has a clear genetic component, with mutations in four genes accounting for ~50% of familial and ~10% of sporadic cases. Several other rarer ALS genes have been identified but their mechanistic role in causing TDP-43 proteinopathy is unknown. Exome sequencing of 699 index familial ALS cases identified mutations in three recently discovered ALS genes: 16 in Tank Binding Kinase 1 (*TBK1*), a kinase involved in inflammation and autophagy; five in Matrin 3 (*MATR3*), a DNA/RNA binding protein; and one in Optineurin (*OPTN*) in a consanguineous Palestinian family with an aggressive form of ALS. The experiments described in this thesis sought to explore the functional impact of these novel ALS-associated variants with the aim of determining their potential pathogenicity and providing insights into disease mechanisms.

Here I show that missense and nonsense *TBK1* mutations can disrupt its kinase activity by interfering with homodimer formation and phosphorylation of its targets IRF3, OPTN and itself, pointing towards a common loss of kinase function mechanism. Furthermore, I show that the novel *OPTN* p.S174X truncation mutation causes a complete loss of OPTN protein expression in homozygous, and haploinsufficiency in heterozygous patient fibroblasts due to nonsense mediated decay. Heterozygotes show a minor increase in the mitochondrial markers TOM20 and COXIV and increased cellular respiration under a stress test but do not present any striking mitophagy or autophagy defects and homozygous fibroblasts are apparently unaffected. Lastly I explore the effects of *MATR3* mutations on protein solubility which was decreased for the p.S85C mutant and that p.R841C forms cytoplasmic inclusions, however no phenotype was elicited for two other mutations.

The findings in this thesis have made a modest contribution to our knowledge of the molecular mechanisms underlying the pathogenesis of ALS, the understanding of which is pivotal in order to develop more effective therapies for this dreadful disease.

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Abbreviations

A

ABIN	A-20 binding inhibitors of NF-kB
AD	Alzheimer's disease
ADA	AdaBoost
ALS	Amyotrophic Lateral Sclerosis
ALS2	Autosomal recessive juvenile-onset form of ALS
ALSdb	ALS data base
ALSoD	Amyotrophic lateral sclerosis online genetics database
ANG	Angiogenin
ANXA11	Annexin A11
ANOVA	Analysis of Variance
AOD	Age of Death
AOO	Age of Onset
AR	Autosomal Recessive
ASO	Antisense oligonucleotide
A-T	Ataxia-Telangiectasia
ATG	Autophagy-Related Genes
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine triphosphate
ATXN2	Ataxin 2

B

BDT	Big Dye Terminator
BMAA	beta-N-methylamino-L-alanine
BRC	Biomedical Research Centre
BSA	Bovine serum albumin

C

C_4G_2	CCCCGG
C9ORF72	Chromosome 9 open reading frame 72
C. Elegans	*Caenorhabditis Elegans*
C-terminal	Carboxy terminal
Ca^{2+}	Calcium
CCD	Coiled Coil Domain
CCD 1/2	Coiled Coil Domain 1/2
CCDS	Consensus Coding Sequence
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain containing 10
CHMPB2	Charged multivesicular body protein 2B
CHO	Chinese Hamster Ovary
CHX	Cyclohexamide
cDNA	Complementary Deoxyribonucleic Acid
CDS	Coding Sequence
CMT	Charcot-Marie-Toooth Disease
CNS	Central Nervous System
COXIV	Cytochrome c oxidase IV
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF	Cerebrospinal Fluid
CO_2	Carbon dioxide
Co-IP	Co-immunoprecipitation
CT	Cycle Threshold
CTD	C-Terminal Domain

D

DAPI	4', 6-diamidino-2-phenylindole
DCTN1	Dynactin 1
ddA	dideoxyadenine

ddC	dideoxycytosine
ddG	dideoxyguanine
$_{dd}H_2O$	Double distilled water
ddNTP	dideoxynucleotides
ddT	dideoxythymine
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Dinucleotide tri phosphates
DPBS	Dulbecco's phosphate-buffered saline
DPR	Dipeptide repeat
dsDNA	double strand DNA
dsRNA	double strand RNA
DUL	Distal Upper Limb
DTT	DL-dithiothreitol

E

E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
ECACC	European Collection of Authenticated Cell Cultures
ECAR	Extracellular Acidification Rate
ECL	Enhanced Chemiluminescence
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELP3	Elongator protein 3
EMG	Elecromyography
ER	Endoplasmic Reticulum

EtBr	Ethidium Bromide
EVS	Exome Variant Server
EWSR-1	Ewing's sarcoma RNA binding protein 1
ExAC	Exome Aggregation Consortium

F

fALS	Familial Amyotrophic Lateral Sclerosis
FBS	Fetal Bovine Serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FDA	Food and drug agency
FIG4	Phosphoinsoited phosphatase
fs	frameshift
FT	Flow-through
FTD	Fronto-temporal Dementia
FUS	Fused in sarcoma

G

G_4C_2	GGGGCC
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gr	Grams
GRCh37	Genome Reference Consortium Human Build 37
GSK3β	Glycogen synthase kinase 3 beta
GTP	Guanine tri-phosphate
GWAS	Genome Wide Association Study

H

HA	Hemagglutinin
HCL	Hydrochloric acid
HD	Huntington's Disease
HEK-293T	Human embryonic kidney cells
hnRNP	Heterogeneous nuclear ribonucleoprotein

HRP	Horse radish peroxidase
HSE	Herpes Simplex Encephalitis
HSV1	Herpes Simplex Virus-1

I

IBMPFD	Paget disease of the bone and frontotemporal dementia
IBD	Inheritance By Descent
ICC	Immunocytochemistry
i.e.	*Id est*
IFNs	Interferons
IFNα/β/λ	Interferon α/β/λ
IgG	Immunoglobulin G
IP	immunoprecipitation
iPSC	Induced pluripotent stem cell
IRF3	Interferon Regulatory Factor 3

K

K	Lysine
Kb	Kilobase
KCL	King's College London
KD	Kinase Domain
kDa	Kilo Dalton
KO	Knock out

L

l	Litre
L	Limbs
LC3	1A/1B-Light Chain 3
LCL	Lymphoblastoid Cell Line
LIR	LC3 interacting region
LIPE	Lipase E

LL	Lower Limb
LMN	Lower Motor Neuron

M

m	Meter
M	Molar (moles/liter)
MAF	Minor Allele Frequency
MAMs	Mitochondria Associated Membranes
MAP2	Microtubule-associated protein 2
MATR3	Matrin 3
Mb	Megabyte
$MgCl_2$	Magnesium chloride
mHtt	mutant Hungtintin
min	Minutes
miRNA	Micro RNA
MN	Motor neuron
MND	Motor Neurone Disease
MNDA	Motor Neurone Degeneration Association
MRI	Magnetic Resonance Imaging
MRS	Membrane Retention Signal
mRNA	Messenger RNA
MOPS	3-(N-morpholino) propanesulfonic acid
MSCs	mesenchymal stem cells
MSc	Master of Science
MSP	Multisystem Proteinopathy

N

N-terminal	Amino terminal
NDP52	Nuclear Dot Protein 52
NEFH	Heavy Neurofilament Subunit

NEHJ	Non Homologous End Joining
NEK1	NIMA-related kinase 1
NEMO	NF-kB modulators
NES	Nuclear Export Signal
NFkB	Nuclear Factor k B
NF	Neurofilament
NFE	Non-Finnish European
NHDF	Normal Human Dermal Fibroblasts
NLS	Nuclear Localising Signal
NMD	Nonsense Mediated Decay
NMJ	Neuromuscular junction
NONO	Non-POU domain-containing octamer-binding protein
NP-40	nonyl phenoxypolyethoxylethanol
NPC	Nucleo Pore Complex
ns	Non significant
NSCs	Neural Stem Cells
NSC-34	Neuroblastoma Spinal Cord 34
NTD	N-Terminal Domain
NTG	Normal Tension Glaucoma

O

OCR	Oxygen consumption rate
OMM	Outer Mitochondrial Membrane
OPTN	Optineurin
OR	Odds Ratio
ORF	Open Reading Frame

P

PBP	Progressive Bulbar Palsy
PBS	Phosphate saline buffer

PCR	Polymerase Chain Reaction
PD	Parkinson’s Disease
PFN1	Profilin 1
Pi	Proteinase Inhibitors
PI(3,5)P2	phosphatidyl-inositol-3,5-biphosphate
PINK1	PTEN-induced putative kinase 1
Pho-STOP	Phosphatase Inhibitors
PLS	Primary Lateral Sclerosis
pM	Pico Moles
POAG	Primary Open Angle Glaucoma
Poly-GA	poly-Gly-Ala
Poly-GP	poly-Gly-Pro
Poly-GR	poly-Gly-Arg
Poly-PA	poly-Pro-Ala
Poly-PR	poly-Pro-Arg
PrLD	Prion-Like Domain
PTPIP51	Protein tyrosine phosphatase-interacting protein-51
PVDF	Polyvinylidene difluoride

Q

qPCR	Quantitative polymerase chain reaction

R

R	Respiratory
RAN	Repeat-associated non-ATG
RAN	RAs-related Nuclear protein
Ref	Reference
RefSeq	Reference Sequence
RF	Random Forest
RRM	RNA Recognition Motif

RNA	Ribonucleic Acid
RIPA	Radio-Immunoprecipitation Assay
RLRs	RIG-I-like receptors
RoH	Regions of shared Homozygosity
ROS	Reactive oxygen species
RPL13A	Ribosomal Protein L13a
RT-PCR	reverse transcriptase polymerase chain reaction

S

S	Spinal
sALS	Sporadic Amyotrophic lateral sclerosis
SCA2	Spinocerebellar ataxia type 2
SDD	Scaffold Dimerization Domain
SDHA	Succinate Dehydrogenase Complex Flavoprotein Subunit A
SDS	Sodium Dodecyl sulphate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide
SEM	Standard error of the mean
Ser	Serine
SETX	Senataxin
SFPQ	Splicing Factor Proline And Glutamine Rich
siRNA	Small Interfering RNA
SMA	Spinal Muscular Atrophy
SNP	Single Nucleotide Polymorphism
snRNA	Small Nucleolar Ribonucleic Acid
SOD1	Superoxide dismutase 1
SPG11	Spatacsin
SQSTM1	Sequestosome 1

T

TAE	tris-acetate-ethylenediaminetetraacetic acid

TAF-15	Tata-binding protein associated factor 2N Thermophilus
Taq	Thermophilus aquaticus
TAR	Trans-Activation Response
TARDBP/TDP- 43	Transactive response DNA-binding protein 43
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TE	Trypsin/ solution ethylenediaminetetraacetic acid
TLR	Toll Like Receptor
TLS	Translated in Liposarcoma
TOM	mitochondrial receptor complex
TOM20	Translocase of Outer Membrane 20
TRIF	TIR-domain-containing adaptor-inducing interferon-β
TRIS	tris(hydroxymethyl)aminomethane
TRIS-HCL	tris(hydroxymethyl)aminomethane-hydrocloric acid
TUBA4A	Tubulin Alpha 4a

U

UBAN	Ubiquitin Binding region of A-20 binding inhibitors of NF-kB proteins and NF-kB modulators
UBQLN2	Ubiquilin 2
UCSC	University of California Santa Cruz Genome Browser
UL	Upper Limb
ULD	Ubiquitin Like Domain
UMN	Upper Motor Neuron
UMN-D	Upper Motor Neuron Dominant
UNTR	Untransfected
UPS	Ubiquitin proteasome system
UV	Ultraviolet

V

V	Volts
VAPB	VAMP Associated Protein B and C
VCP	Valosin-containing protein
VCF	Variant Calling File

W

WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WT	Wild Type

Z

ZF	Zinc Finger

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Chapter 1 – Introduction

1.1 Overview of Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) or Motor Neuron Disease (MND), also known as Lou Gherig's disease, is an adult onset neurodegenerative disorder that predominantly targets the upper and lower motor neurons, which causes progressive muscle wasting and weakness and stiffness due to spasticity. This condition was considered a muscular dysfunction until 1869, when Jean-Martin Charcot (1825–1893) correctly identified that degeneration of spinal cord motor neurons caused muscular 'amyotrophy' and 'lateral sclerosis' (scarring) of the corticospinal tracts from degeneration of upper motor neurons, and gave it the name amyotrophic lateral sclerosis (Katz, Dimachkie and Barohn, 2015).

ALS patients can initially present with symptoms in either limb or bulbar (throat) territories. Around 65% of the cases can be classified as being of *spinal* onset affecting the limbs, whereas 30% of the cases have a bulbar onset causing dysarthria, dysphagia or both, and 5% present with breathless due to diaphragm weakness (Kiernan et al. 2011; Zufiría et al. 2016). Classically ALS patients show similar symptoms such as: muscle wasting, weakness and fasciculation, caused by the degeneration of lower motor neurons, and spasticity, hyper-reflexia and an extensor plantar response (Babinski sign), caused by the upper motor neuron degeneration (Taylor, Brown and Cleveland, 2016). Survival rate is generally 3-5 years from diagnosis, typically by respiratory failure (Shaw, Al-Chalabi and Leigh, 2001).

The prevalence of ALS is around 5 cases per 100,000 people with a lifetime risk for men of 1 in 350, compared to 1 in 400 for women. These statistics are derived from analysing populations of European origin as these communities have been the focus of most epidemiological studies (Al-Chalabi and Hardiman, 2013). The average age of onset is 55 years old (Taylor et al. 2016), however, in rare cases the disease onset can be considered early onset (<45 years old) or juvenile (<25 years old) (Turner *et al.*, 2012).

Despite this disease being recognised for almost 150 years and a considerable amount of progress in terms of research, there is still no effective treatment for ALS. The only compound currently used for treatment in Europe and USA is Riluzole, a glutamate release inhibitor, that has been shown to increase survival by only a few months (Jardin et al. 2010; Lacomblez et al. 1996; Riviere et al. 1998).

In ~10% of ALS cases other family members have been affected by ALS or a linked form of frontotemporal dementia (FTD) and are, therefore, described as 'familial ALS' (fALS). The majority of these cases show a Mendelian inheritance pattern of the defective gene (Byrne *et al.*, 2011), most often with a dominant trait and high penetrance (Taylor et al. 2016). To date more than 50 genes have been found to be involved in the aetiology of fALS. Among these, four genes account for ~60% of the fALS cases: *C9orf72* (~25%), *SOD1* (~20%), *FUS* (~4%), *TARDBP* (~5%). *C9ORF72* expansion tends to cause disease late in life, representing 50% of cases with an age of onset > 40 years old while *FUS* mutations accounts for 30% of the cases with age of onset < 40 years old (Millecamps *et al.*, 2012).

The remaining ~90% of ALS cases that do not have a family history of ALS or FTD are described as being sporadic ALS (sALS) cases. The lack of family history does not exclude a genetic contribution as most genes are incompletely penetrant and of late onset (particularly *C9ORF72*) and fast progression, making the task of tracking familial history particularly challenging. In some cases, it is possible to see an overlap between genes identified in fALS and genes linked to sALS. For instance: 4% of sALS are associated with missense mutations in SOD1 (Gamez *et al.*, 2006) and over 5% of the cases are linked to *C9ORF72* expansion (Cooper-Knock *et al.*, 2012).

ALS is associated, clinically and pathologically, with another adult-onset neurodegenerative disease, frontotemporal dementia (FTD), the second most common cause of dementia presenting under age of 65 (McKhann *et al.*, 2001; Forman *et al.*, 2006). Many of the pathological processes that lead to a loss of motor neurons in ALS are also implicated as causing the loss of cortical neurons in the frontal and temporal

lobes, which are a feature of FTD. This corresponds, clinically, with the cognitive impairment that 50% of ALS patients experience during the course of their disease. Around 20% of the ALS cases meet the criteria for a diagnosis of FTD (Taylor et al. 2016).

1.2 Clinical features

ALS presents a very heterogeneous spectrum of clinical features. The diagnosis of ALS is based on a history of progressive muscle weakness and a combination of signs indicating the degeneration of upper motor neurons (UMN, originating in the frontal cortex and projecting to neurons in the spinal cord) and lower motor neurons (LMN, originating in the spinal cord and innervating skeletal muscle fibres) (Talbot, 2009) (Figure 1.1). Typical ALS can be divided into two main groups:

1. Limb-onset ALS (~65%): This presents with fasciculation in the initial phase of the disease, impairment of the limb movement and muscle wasting and weakness (Talbot, 2009).

2. Bulbar-onset ALS (~30%): This typically presents with difficulties in speaking and swallowing with signs of tongue atrophy. These patients often develop limb symptoms later in the disease (Kiernan *et al.*, 2011) and usually have a worse prognosis compared to limb-onset ALS patients (Mortara *et al.*, 1981).

There are two more phenotype that are, sometimes, associated with ALS and can develop into classic ALS but have, generally, a better prognosis. It is, however, important to distinguish these phenotypes from classic ALS (Talbot, 2009):

1. Primary Lateral Sclerosis (PLS): This is predominantly an upper motor neuron disease usually presenting with stiffness and modest weakness in the legs as primary symptoms, with a spastic increase in tone, exaggerated reflexes and an extensor plantar response. This group have the better prognosis with survival usually ≥5 years and may be much longer (Gordon *et al.*, 2006).

2. Progressive Muscular Atrophy (PMA): This presents with progressive muscle wasting due to lower motor neuron degeneration (Gordon *et al.*, 2006). Only 4% of MND patients show this form of MND (Wijesekera *et al.*, 2009) with survival rate as high as decades (Talbot, 2009). However, 40% of PMA patients will develop UMN symptoms within 18 months and therefore reclassified as having ALS (Jeldican *et al.*, 2007).

ALS is an aggressive disease and it displays a short survival time in most of the cases. Approximately 50% of patients will die within 30 months from symptoms onset, around 15-20% will survive 5 years and only a small percentage will live more than 10 years from diagnosis (Talbot, 2009). Death usually occurs due to respiratory failure due to weakness of thoracic and diaphragmatic muscles.

Interestingly, there is no strict association between specific gene mutations and particular clinical features. However, mutations in the *Fused in Sarcoma* (*FUS*) gene are associated with an early onset of ALS upper limb and rapid progression (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009; Bäumer *et al.*, 2010; MacKenzie *et al.*, 2011; Mackenzie *et al.*, 2012). Even patients who are carriers of the same gene, may display differences in the manifestation of the disease, for example: mutations in *Cu/Zn Superoxide Dismutase 1* (*SOD1*), the first gene to be associated with ALS (Rosen *et al.*, 1993), can have different phenotypes: p.A4V in the heterozygous state shows an aggressive and very rapid disease progression (Yim *et al.*, 1997) whereas people heterozygous for p.D90A *SOD1* mutation are usually asymptomatic and even homozygous patients develop slowly progressive disease (Juneja *et al.*, 1997).

Around 20-50% of ALS patients show cognitive impairment symptoms such as personality change, decreased verbal fluency, irritability and failure to perform tasks that require frontal executive functions. These symptoms are recognised as a mild manifestation of FTD. The clinical and pathological overlap between ALS and FTD is such that it is now accepted that the two conditions represent phenotypic ends of a common spectrum (Taylor et al. 2016). 95% of ALS cases have TDP-43 positive

ubiquitinated inclusions in their spinal motor neurons, which are also present in cortical neurons of the frontal and temporal lobes of 95% of tau-negative FTD cases (Neumann *et al.*, 2010).The remaining 5% of FTD patients have cytoplasmic *FUS* even in the absence of FUS mutations, associated with FUS inclusions in spinal motor neuron in ALS. Interestingly, this feature has also been shown by patients with familial ALS (Vance *et al.*, 2009; Neumann *et al.*, 2010; King *et al.*, 2015). Pathological overlap between ALS and FTD has also been observed in the hexanucleotide repeat GGGGCC (G_4C_2) expansion in chromosome 9 open reading frame 72 (*C9ORF72*), which is responsible for 20-50% of fALS and 10% of sALS and FTD cases (Dejesus-hernandez *et al.*, 2011; Renton *et al.*, 2011), where the classical pathology involves TDP-43 negative p62 positive inclusions in the cerebellum, frontal and temporal lobes (Al-Sarraj *et al.*, 2011). This overlap has been confirmed by voxel-based morphometry Magnetic Resonance Imaging (MRI), where a bilateral atrophy of the motor and premotor cortex can be observed in patients with ALS and FTD, although patients with FTD-ALS show a greater frontotemporal atrophy than those with ALS alone (Morita et al. 2006; Neumann et al. 2010).

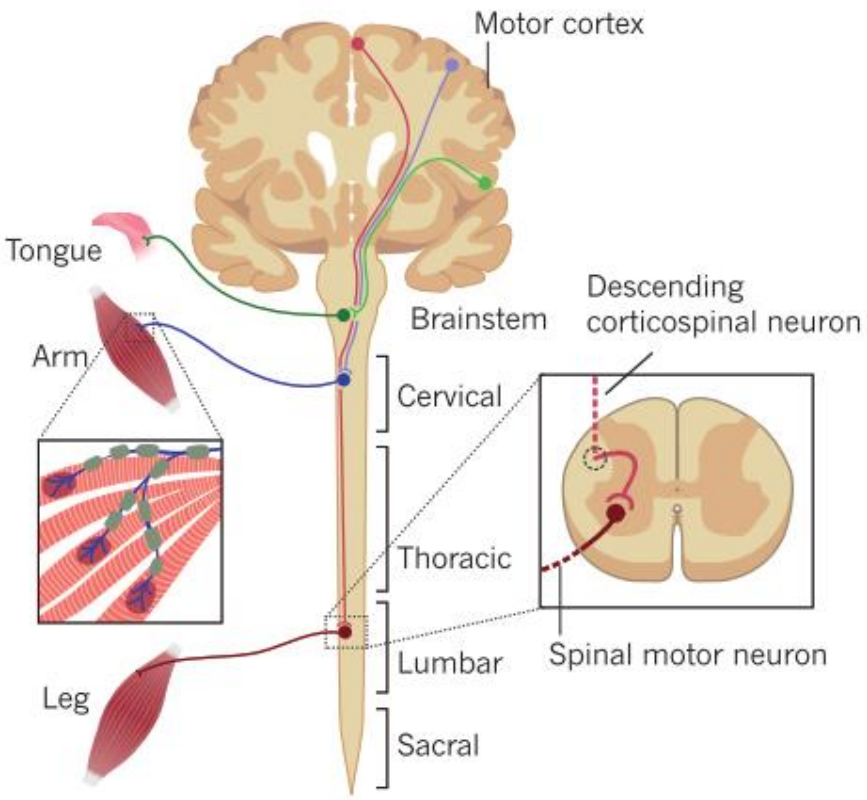


Figure 1.1 – Anatomical representation of the motor system. (Adapted from Taylor et al. 2016).

1.3 Diagnosis

There is no existing diagnostic test for ALS. Therefore, the diagnosis is dependent on clinical evaluation together with negative laboratory tests for diseases with similar symptoms. Electro diagnostic studies are, currently, the most reliable way to confirm a loss of lower motor neurons (fasciculation or spontaneous denervation discharges) and exclude peripheral neuropathy or myopathy (Daube, 2000). Magnetic Resonance Imaging (MRI) of brain and spinal cord is also used to rule out structural disorders such as spinal cord and/or nerve root compression due to bone and/or disc degeneration, inflammation, syringommyelia and tumours (Traynor and Ghasemi, 2000; Turner *et al.*, 2009). Lastly, DNA tests for an expansion mutation in the androgen receptor can be used to diagnose Kennedy's disease, which can mimic ALS (Shaw, Al-Chalabi and Leigh, 2001).

Clinicians base their diagnosis mainly on the combination of UMN and LMN signs in one or more region in the absence of an alternative diagnosis being identified by the tests described above. In 1991 the World Federation of Neurology developed The El Escorial criteria (Brooks *et al.*, 1994), which was later revised in 1998 and in 2008 to acknowledge advances in laboratory testing (de Carvalho *et al.*, 2008; Schrooten *et al.*, 2011). The criteria are listed below:

1. Definite ALS: UMN and LMN signs in three areas of the body (bulbar, arm, leg).

2. Probable ALS: UMN and LMN involvement in two regions with at least some UMN signs rostral to LMN signs.

3. Probable ALS—laboratory supported: UMN signs in one or more regions and LMN degeneration evidence defined by electromyography in at least two regions.

4. Possible ALS: UMN and LMN involvement in one region. UMN signs in two or more regions. UMN signs and LMN signs in two regions with no UMN involvement rostral to LMN signs.

1.4 Aetiology

The aetiology of all ALS cases is yet to be fully determined but a combination of environmental and genetics factors have been implicated: male gender, advanced age and genetic history of ALS (Ingre *et al.*, 2015). Hereditability research has reported that about 60% of the risk of ALS is determined by genetic factors and 40% is caused by environmental factors (Al-Chalabi et al. 2010). Nonetheless, the correlation between genetic and environmental risk with phenotype remains unexplained (Al-Chalabi and Hardiman 2013).

1.4.1 Environmental factors

A number of epidemiological and population based studies have suggested that environmental factors might interact with genetic factors and modify the aetiopathogenic

process of ALS. In 2014 a population-based study suggested that ALS could be a multi-step process, more specifically a mathematical model revealed that a total of 6 environmental and/or genetic factors are needed in order for ALS to occur (Al-Chalabi et al. 2014). The first association of ALS with environmental factors was found in Guam, when indigenous people showed a particularly high incidence of ALS in the population. This was attributed to the accumulation in the CNS of a neurotoxin: the beta-N-methylamino-L-alanine (BMAA) (Banack and Cox, 2003; Bradley and Mash, 2009). Another environmental factor related to ALS is heavy metal exposure (Ingre *et al.*, 2015), indeed higher levels of lead were found in the blood and bones of ALS patients compared to controls (Kamel *et al.*, 2002; Fang *et al.*, 2010). Manganese is known to have neurotoxic effects with manifestation of motor impairment (Sjögren *et al.*, 1996) and was also found to be abundant in the cerebral spinal fluid (CSF) of ALS patients (Roos *et al.*, 2012). Involvement of pesticides has also been suggested (Gunnarsson *et al.*, 1992; McGuire *et al.*, 1997) and confirmed more recently by meta-analysis studies (Kamel *et al.*, 2012; Malek *et al.*, 2012). However, environmental risk factors are very complicated to identify as the exposure to any environmental factor is rarely quantifiable and highly variable.

1.4.2 Genetics

Approximately 10% of ALS cases show familiar history (fALS), with at least one other affected member in the family, the remaining 90% of cases are apparent sporadic ALS (sALS) (Rowland, Hneider and Shneider, 2001). The past 30 years of research in ALS have seen an exponential growth in the number of ALS associated genes, discovered initially through classical linkage and association studies and more recently through whole exome sequencing (WES) and whole genome sequencing (WGS). However, linkage analysis in ALS kindreds is problematic as it is a late onset disorder with short survival and incomplete penetrance and large DNA collections from suitable pedigrees are rare. WES and WGS are novel and promising techniques that have already identified important genes in ALS. To date, over 50 genes have been reported to be linked to ALS

pathogenesis accounting for ~60% of familial ALS cases (Figure 1.2) and ~ 10% of sporadic ALS cases (van Es *et al.*, 2017), with 50% of the fALS being caused by the four main genes: *SOD1, C9ORF72, TARDBP* and *FUS/TLS* (Ling, Polymenidou and Cleveland, 2013). Considering that the genes discovered in fALS are often the same as the ones implicated in sALS, a strict classification of ALS cases into sporadic and familial ALS is an oversimplification (Al-Chalabi *et al.*, 2010). Most of the studies published until now suggest an overlap between the genetic architecture of these two categories (van Es *et al.*, 2017). The ALS risk for a first order family member of a sALS patient is eight-fold higher compared to the population risk at age 85, raising their risk to 2.4% (Hanby *et al.*, 2011).

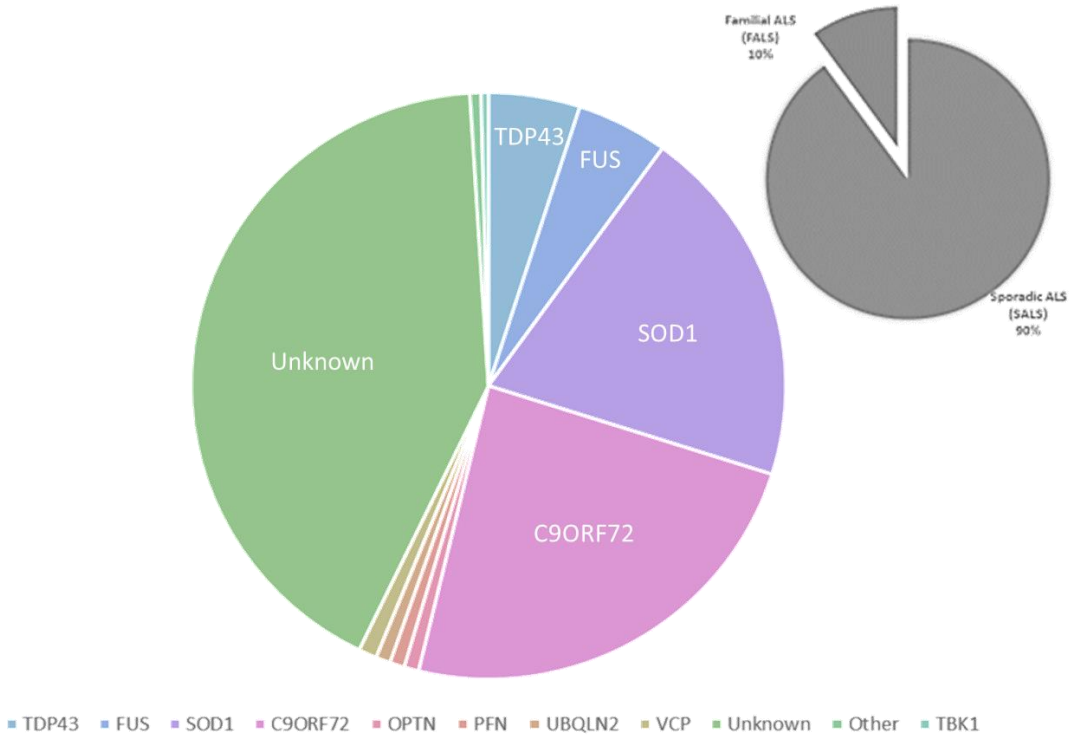


Figure 1.2 – Percentage of mutated genes in familial ALS.

To unravel the complex genetics of this disease, a large scale GWAS project has been launched in 2012 called Project MinE (https://www.projectmine.com/), aiming to genome

sequence 15000 ALS cases and 7500 controls. This project is predicted to be finished by 2018. Here will follow a description of all the major genes discovered in ALS to date.

1.4.2.1 Principal genes

This section will focus on the four main genes that together represents ~20% of all ALS cases (Al-Chalabi *et al.*, 2012; White and Sreedharan, 2016).

1.4.2.1.1 Superoxide Dismutase 1 (*SOD1*)

SOD1 was the first gene to be linked to ALS. Disease associated mutations in this gene were discovered through linkage studies in 1993 when Rosen and colleagues identified 11 missense mutations implicated in ALS (Rosen *et al.*, 1993). To date over 190, mostly missense, mutations were found spread along the 153 codons of *SOD1* and account for ~20% of fALS (ALS data base (ALSdb, http://alsdb.org); van Es et al. 2017) and 5% of apparently sporadic cases (Kiernan *et al.*, 2011). Mutations are almost exclusively found within the exons, suggesting that they might gain their toxicity through the disruption of the protein structure or function (Sangwan and Eisenberg, 2016). *SOD1* encodes for a protein (SOD1) that catalyses the conversion of superoxide radicals in hydrogen peroxide, through cyclical reduction and oxidation of copper (Zelko, Mariani and Folz, 2002; Pasinelli and Brown, 2006). This protein is ubiquitous among human cells and mostly cytoplasmic. In order to operate its function, SOD1 needs to homodimerise, with each subunit binding one atom of zinc and one of copper. Many mutations found in ALS cases do not affect its catalytic activity, so loss of catalytic function is not a requirement for pathogenicity (Pasinelli and Brown, 2006). The mechanism of pathogenicity seems to be due to a toxic gain of function caused by a change in the amino acid sequence, which causes SOD1 misfolding and aggregation in the cell (Pasinelli and Brown, 2006).

Interestingly, from a clinical point of view, patients who carry different *SOD1* mutations, can present in different ways. Most mutations are heterozygous and are passed down generations in an autosomal dominant pattern of inheritance. However, exceptions do exist, for example the p.D90A mutation causes ALS only when both alleles are mutated.

This mutation is heterozygous in 2-3% of the Scandinavian population, showing high penetrance when homozygous (Andersen *et al.*, 1996; Robberecht *et al.*, 1996). Symptoms caused by this mutation are very distinctive and show a slowly progressive form of ALS, with corticospinal signs and average survival of about 10 years. *SOD1* pathology is rarely associated with dementia, however, patients homozygous for the mutation p.D90A show cognitive impairment in the later stages of the disease, probably due to the prolonged duration of the illness (Turner et al. 2005). Interestingly, some individuals that carry this mutation in heterozygosity outside the Scandinavian population develop classic ALS with survival of three to five years (Pasinelli and Brown, 2006). It has been proposed that the North Scandinavian population might carry some neuroprotective allele that would, somehow, weaken the toxicity of the mutation (Soares *et al.*, 2004). The p.A4V *SOD1* mutation is the most abundant in North America, representing ~50% of the familial ALS cases. It presents with a predominantly lower motor neuron involvement and is rapidly progressive with mean survival of one year (Cudkowicz *et al.*, 1997).

Many transgenic mouse models have been created in order to better understand the molecular mechanisms whereby mutated SOD1 causes motor neuron degeneration in ALS (Pasinelli *et al.*, 2004). These models have also been used to test compounds that could be used in clinical trials, however, this approach has been criticised by many (Ludolph *et al.*, 2007). Mouse models have confirmed that knock down of wild-type SOD1 causes no phenotype in mice (Bruijn *et al.*, 1998) whereas the expression of many SOD1 mutants leads to motor neuron degeneration. Collectively the evidence indicates that *SOD1* might cause the disease through a toxic gain of function (Sangwan and Eisenberg, 2016).

Although there is no evidence of the transmission of ALS between individuals, a prion-like behaviour has been hypothesised for mutant SOD1. This theory was supported by some evidence showing that SOD1 is secreted extracellularly *in vitro* (Münch, O'Brien, and Bertolotti 2011; Sundaramoorthy et al. 2013; Turner et al. 2005). Furthermore,

homogenates of mouse spinal cord has been found to cause SOD1 aggregation in *vitro* (Chia *et al.*, 2010) and, if injected in mice, to induce the pathology, which spreads along the spinal cord (Grad *et al.*, 2011, 2014, Ayers *et al.*, 2014, 2016). This theory could also explain the spread of clinical symptoms between anatomically connected areas to the region of onset (Sangwan and Eisenberg, 2016).

Over 25 years of investigation of *SOD1* ALS have revealed that mutant SOD1 aggregation is toxic to multiple cellular systems including mitochondria function, axonal transport and proteostasis (Al-Chalabi *et al.*, 2012). Therapies targeting the mutant RNA and protein are showing promise. Gene silencing through antisense oligonucleotide has been shown to reduce the level of mutant SOD1 and to be well tolerated in phase 1 clinical trials (Miller *et al.*, 2013; Nizzardo *et al.*, 2016). Furthermore, an immunisation approach has also been explored and it has shown to be marginally effective in mouse models (Urushitani, Ezzi and Julien, 2007; Gros-Louis *et al.*, 2010; Liu *et al.*, 2012). Mutant SOD1 cases, however, account for only ~3% of all ALS and they do not show the classical pathology of TDP-43 cytoplasmic inclusions, present in the vast majority of sporadic and familial ALS cases (Mackenzie *et al.*, 2007). Therefore, therapies shown to be effective in mutant *SOD1* cellular and animal models of ALS may not be effective in most ALS cases.

1.4.2.1.2 TAR DNA-binding protein (*TARDBP*)

A major breakthrough in ALS research was the discovery that TDP-43 (encoded by *TARDBP*), an RNA binding protein, represented a major component of the ubiquitin-positive inclusions found in the spinal cord of almost all ALS and tau-negative FTD patients (Neumann *et al.*, 2006). TDP-43 is a protein composed by 414 amino acids, carrying two RNA recognition motifs (RRM) and a low complexity glycine-rich domain at the C-terminus end of the protein (Kato *et al.*, 2012; King, Gitler and Shorter, 2012). To date over 50 *TARDP*, predominantly missense mutations, have been associated with ALS, and account for ~5% of fALS and ~1% of sALS (www.alsdb.org; Sreedharan et al. 2008), and represent 1-2% of total ALS cases (Sreedharan *et al.*, 2008; Renton, Chiò

and Traynor, 2013). The distribution of mutations among different populations is mostly even with some exceptions. For instance, the p.A382T variant is enriched in the Sardinian population (Chiò *et al.*, 2011). Most of the mutations cause single amino acid substitutions in the glycine-rich domain, at the C-terminal region, which is involved in the binding of TDP-43 to the other ribonucleoprotein (Renton, Chiò and Traynor, 2013) as well as in protein-protein interaction (Buratti *et al.*, 2005).

TDP-43 was originally identified as a transcriptional repressor of the trans-activation response (TAR) element of the HIV virus (Ou *et al.*, 1995). It is predominantly nuclear, where it binds and regulates RNA transcription, splicing and transport (Buratti and Baralle, 2012). Furthermore, it shuttles between the nucleus and the cytosol where it delivers RNA to the ribosomal machinery in the cytoplasm and in the axodendritic arborisation. TDP-43 binds particularly to UGUG RNA motifs dictating the inclusions or exclusion of specific exons (Buratti and Baralle, 2001; Tollervey *et al.*, 2011). A great deal of attention has focussed on the role TDP-43 plays in alternative splicing, a function that is disturbed when TDP-43 is sequestered into cytoplasmic aggregates and unable to enter the nucleus (Freibaum *et al.*, 2010; Polymenidou *et al.*, 2011; Tollervey *et al.*, 2011). TDP-43 has also been found in RNA granules, promoting anterograde axonal transport and regulating the translation of a subgroup of mRNAs (Wang *et al.*, 2008; Alami *et al.*, 2014). TDP-43 is also recruited to stress granules, membrane-less cytosolic RNA-protein clusters that arise in response to oxidative, osmotic and heat stressors (Li *et al.*, 2013). It is hypothesised that TDP-43 is present in high concentrations in stress granules where it misfolds to form toxic oligomer ‘seeds’, which initiate further aggregation and inclusion formation. Thus the stress granule has been labelled “the crucible of aggregation” (Li *et al.*, 2013).

The most common feature of TDP-43 associated pathology is the presence of TDP-43 in insoluble inclusions in the cytosol, which entails a loss of nuclear TDP-43 and the formation of TDP-43 C-terminal fragments (20-25 and 30 kDa). Furthermore, aberrant phosphorylation and ubiquitination of TDP-43 inclusions are evident in upper and lower

motor neurons as well as in the prefrontal cortex in ALS-FTD cases (Geser, Lee and Trojanowski, 2010; Mackenzie, Rademakers and Neumann, 2010). Interestingly, 95% of all ALS patients present TDP-43 pathology with the exception of mutant *SOD1* and *FUS* cases (Mackenzie *et al.*, 2007; Tan *et al.*, 2007), with indistinguishable features between patients positive or negative for mutation in TDP-43 itself (Pamphlett *et al.*, 2009).

The underlying mechanism driving neurodegeneration due to TDP-43 aggregation is still to be determined. In addition to the loss of splicing function described above, TDP-43 mutations cause the protein to be unstable and aggregate in the cytoplasm, which may be directly toxic to motor neurons (Johnson *et al.*, 2009; Kabashi *et al.*, 2009). Recent research has shown that mutant TDP-43 can disrupt the axonal transport of RNA (Alami *et al.*, 2014) and cause mitochondria dysfunction (Wang *et al.*, 2016), which is common to the effects of mutant SOD1.

Overexpression of TDP-43 to 2.5 fold in mice showed an aggressive phenotype with early onset and short survival (2 months from symptom onset) with evidence of TDP-43 pathology (Y. Xu *et al.*, 2011). However, these mice are not a very good model as they die prematurely because of intestinal complication (Esmaeili *et al.*, 2013; Hatzipetros *et al.*, 2014). Mouse models that express p.A315T and p.G348C mutations, show age related motor deficit symptoms but no paralysis or early death (Picher-Martel *et al.*, 2016). In 2015 a study reported that double transgenic mice for humanTDP-43^{WT} and humanTDP-43^{Q331K} display a very early onset limb paralysis with death occurring at 8-10 weeks of age (Mitchell *et al.*, 2015). Single p.Q331K transgenic mice, on the other hand, presented with mild motor dysfunction at three months of age which progressed slowly over 24 months when they showed reduced endurance on the rotaroad, a device used to test mice locomotion. These mice show classic TDP-43 pathology presenting most of ALS related features (Mitchell *et al.*, 2015). However, the overexpression of human TDP-43 in mice entails a reduction in the endogenous level of mouse TDP-43 (Arnold *et al.*, 2013), making it hard to differentiate between a loss of endogenous mouse TDP-43 function or a gain of function caused by overabundant human TDP-43.

Overexpression of wild type and mutant TDP-43 has also been shown to cause aggregation in *Caenorhabditis Elegans* (*C*. *Elegans*) with motility impairment and degeneration pattern that was aggravated in mutants compared to wild type (Ash *et al.*, 2010; Liachko, Guthrie and Kraemer, 2010). Furthermore, injections of mutant TDP43 in zebrafish embryo show evidence of motor axonopathy (Kabashi *et al.*, 2009; Laird *et al.*, 2010).

The discovery of TDP-43 has highlighted the importance of RNA processing in ALS pathogenesis, which was confirmed by the discovery of another RNA binding protein, FUS described below, prompting further investigation on how mutations in these genes might affect RNA metabolism (Lagier-Tourenne *et al.*, 2012).

1.4.2.1.3 Fused in Sarcoma (FUS)

Fused in Sarcoma or Translated in Liposarcoma (*FUS/TLS*) was initially reported as an oncofusion protein involved in liposarcoma (Crozat *et al.*, 1993; Rabbitts *et al.*, 1993). Mutations in *FUS* were later identified in familial ALS by two independent studies (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). Since then, over 70 *FUS* variants have been reported in ALS patients, accounting for ~4% of the fALS and ~1% of the apparently sporadic ALS cases (Picher-Martel *et al.*, 2016). Similar to *TDP-43*, mutations discovered in *FUS* are mainly missense variants, although a few truncation, deletion and insertion variants have also been reported. These cluster in two sites at the extreme carboxylate terminus of the protein and in the low complexity domain (Lagier-Tourenne, Polymenidou and Cleveland, 2010; Andersen and Al-Chalabi, 2011).

Many studies have shown that mutations in *FUS* cause an early onset (age <40 years old), sometimes even juvenile (<25 years), and rapid disease progression (Waibel *et al.*, 2013). ALS patients with FUS mutations have a predominantly LMN onset with rare cognitive symptoms (Lattante, Rouleau and Kabashi, 2013) and truncation mutations show a more aggressive phenotype compared to missense mutations (Picher-Martel *et al.*, 2016).

FUS pathology is varied across the mutation spectrum and even within patients carrier for the same mutation (Kapeli, Martinez and Yeo, 2017). A common pathological feature of FUS is the mislocalisation of the protein, usually nuclear, to the cytosol either as inclusions or diffused smaller aggregates. FUS in ALS has also been reported to aggregate in stress granules and form ubiquitin-negative inclusions (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). Interestingly, cytoplasmic FUS inclusions have also been described in TDP-43 and tau negative FTD (Neumann, Rademakers, *et al.*, 2009) and Huntington's disease (Doi *et al.*, 2008). However, no *FUS* mutations have been identified in any of the FTD-FUS cases (Neumann, Roeber, *et al.*, 2009; Urwin, Josephs, *et al.*, 2010; Rohrer *et al.*, 2011; Snowden *et al.*, 2011). Furthermore in these cases, FUS accumulates with TAF1 and EWS proteins and Transportin1, which are absent from mutant FUS inclusions implying a different basis for inclusion formation (Troakes *et al.*, 2013).

Physiologically, FUS is involved in almost identical RNA processing roles as TDP-43, including: the regulation of transcription, alternative splicing and mRNA trafficking (Fujii and Takumi, 2005; Lagier-Tourenne *et al.*, 2012). FUS targets a large spectrum of RNA sequences. Among these, FUS has been shown to bind to the intronic regions of pre-mRNAs, suggesting it might play a role in splicing (Lagier-Tourenne *et al.*, 2012; Rogelj *et al.*, 2012).

The molecular mechanisms underlying the pathogenicity of FUS mislocalisation in ALS are unclear. Studies in mice seem to agree that the over-expression of mutant FUS is sufficient to cause motor neuron degeneration (Nolan, Talbot and Ansorge, 2016), whereas FUS knock out murine models show late neonatal or early postnatal death. Furthermore, selective knock out of FUS from the nervous system does not appear to cause any neurodegenerative phenotype (Sharma *et al.*, 2016). Evidence seem to agree that FUS pathogenicity is associated with its ability to bind RNA and that it might depend on FUS-acquired toxic functions, which could impair RNA and protein homeostasis, or promote toxic aggregation (Kapeli, Martinez and Yeo, 2017). An example of this is

FUS's property to bind to its own intron seven on FUS mRNA and self-regulate its own expression, producing an alternatively spliced form of FUS that will be degraded by nonsense mediated decay (Zhou *et al.*, 2013). FUS variants fail to operate this function, resulting in an accumulation of the protein that might be toxic for the cell.

Most of the mutations found in FUS map at the carboxyl terminus of the protein, where the Nuclear Localisation Sequence (NLS) localise. At the N terminal FUS presents a QGSY-rich and a glycine rich prion like domain, which when mutated gives FUS aggregation-prone properties that are exacerbated by some of the FUS mutations reported in ALS such as p.R521C and p.P525L (Cushman *et al.*, 2010). Mutations in the NLS, on the other end, can impair FUS ability to enter the nucleus, making wild type FUS a target for mutant FUS, which might sequester it in stress granules (Vance *et al.*, 2013).

1.4.2.1.4 C9ORF72

Another important discovery in the genetics of ALS was the identification of an extensive hexanucleotide repeat expansion in intron 1 of the chromosome 9 open reading frame 72 (*C9ORF72*) gene. The locus of this expansion on Chromosome 9p21 was linked to Familial ALS and FTD in 2006 by two independent studies (Morita *et al.*, 2006; Vance *et al.*, 2006) and subsequently in GWAS of sporadic ALS (van Es *et al.*, 2009; Laaksovirta *et al.*, 2010; Shatunov *et al.*, 2010) and TDP-43 positive FTD (Van Deerlin *et al.*, 2010). In 2011, the hexanuclotide repeat expansion GGGGCC (G_4C_2) was discovered to be causative of ALS (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011; Gijselinck *et al.*, 2012). This expansion accounts for ~80% of familial ALS-FTD cases, 20-50% of fALS, 5-20% of sALS and 10-30% of FTD cases, making it the most prevalent genetic cause of ALS and FTD to date (Boeve *et al.*, 2012; Chiò *et al.*, 2012; Cooper-Knock *et al.*, 2012; Hsiung *et al.*, 2012; Mahoney *et al.*, 2012; Simón-Sánchez *et al.*, 2012; Snowden *et al.*, 2012; Smith *et al.*, 2013). Approximately 90% of healthy individuals carry <10 repeat, whereas ALS and FTD cases have been found to harbour hundreds or even thousands G_4C_2 repeats in this locus.

The role of the G_4C_2 expansion in the physiopathology of ALS and FTD is still not clear. *C9OFR72* expansion carriers show higher incidence of bulbar-onset ALS and cognitive impairment in 50% of the cases. These patients often show early onset with fast disease progression (Byrne *et al.*, 2012; Chiò *et al.*, 2012; Millecamps *et al.*, 2012). This is accompanied by upper and lower motor neuron degeneration and TDP-43 positive inclusions (Cooper-Knock *et al.*, 2012). These are usually observed in the brain and the spinal cord of these patients. Moreover, p62 and Ubiquilin 2 positive nuclear and cytoplasmic inclusions are also observed in the granular and molecular layers of the cerebellum and these inclusions are TDP-43 negative (Brettschneider *et al.*, 2012). Additionally, RNA foci were also identified in the frontal cortex, hippocampus and cerebellum of patients carrier for *C9ORF72* expansion (Dejesus-hernandez *et al.*, 2011). Interestingly, some of these cases have been reported to show other neurodegenerative disease symptoms, more typical of Parkinsonism and Huntington's disease (Cooper-Knock *et al.*, 2012; Wilke *et al.*, 2016).

In the last few years of research on *C9ORF72* repeat expansions, many pathogenic molecular mechanism have been proposed, making the topic particularly controversial. The physiologic function of *C9ORF72* coded protein is to date unknown, resulting in a limited understanding of how this expansion might cause the disease. Three moelcular mechanisms have been proposed until now:

1. Loss of function of C9ORF72 (haploinsufficiency) (Ciura *et al.*, 2013)
2. Gain of RNA toxicity (Fratta *et al.*, 2013)
3. Repeat associated non-ATG translation (Zu *et al.*, 2010)

Haploinsufficiency would derive from the unsuccessful translation of mRNA carrying the large expansion. This hypothesis seems unconvincing as no other mutations have been identified on the same gene and homozygosity of this variant in human does not exacerbate the disease manifestation (Fratta *et al.*, 2013). Furthermore, knock out of the

whole gene in mouse does not show any motor neuron degeneration, suggesting that haploinsufficiency is unlikely to be a major cause (Koppers *et al.*, 2015).

RNA foci, localised in the neuronal nucleus and occasionally in the cytoplasm, were identified in *C9ORF72* post mortem brain and spinal cord tissues as well as in patient derived fibroblast and lymphoblast lines (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011; Donnelly *et al.*, 2013; Gendron *et al.*, 2013; Lagier-Tourenne *et al.*, 2013; Mizielinska *et al.*, 2013; Zu *et al.*, 2013). Furthermore, G_4C_2 repeats have been reported to form highly stable G-quadruplex structure (four stranded helical secondary structures that can form in guanine-rich sequences and are stabilised by Hoogsteen hydrogen bonding) as well as R-loops (hybrid structures formed by DNA and RNA) *in vitro* (Fratta *et al.*, 2012; Reddy *et al.*, 2013, 2014; Haeusler, Donnelly and Rothstein, 2016). The RNA gain of function theory states that RNA foci might be able to sequester important proteins involved in RNA metabolism such as hnRNPH, SR2, SC35, ALYREF, ADARB2 or hnRNP-A1, which have been found to localise with the RNA foci in a number of studies (Donnelly *et al.*, 2013; Lee *et al.*, 2013; Mori, Lammich, *et al.*, 2013; Sareen *et al.*, 2013; Cooper-Knock *et al.*, 2014, 2015; Haeusler *et al.*, 2014). The G-quadruplex and R-loop structure could, on the other hand, interact with nucleolar proteins causing nucleolar stress (Haeusler *et al.*, 2014). This hypothesis was confirmed by a number of studies using neuronal cell lines, *Drosophila Melanogaster* and *Zebrafish* (Lee *et al.*, 2013; Xu *et al.*, 2013; K. Zhang *et al.*, 2015; Rossi *et al.*, 2015; Jiang *et al.*, 2016; Y. Liu *et al.*, 2016). However, there are studies in transgenic fly and mouse models as well as induced pluripotent stem cells (iPSCs), which do not confirm this theory (Mizielinska *et al.*, 2014; O'Rourke *et al.*, 2015; Peters *et al.*, 2015; Tran *et al.*, 2015), indicating that RNA foci alone might not be sufficient to cause neurodegeneration.

G_4C_2 repeats were initially reported to go through Repeat Associated Non-ATG translation (RAN translation) by two independent studies (Mori et al. 2013; Zu et al. 2013). The hexanucleotide repeat is translated from the forward and reverse RNA strand to generate five different dipeptide proteins (DPRs): poly-Gly-Ala (GA), poly-Gly-Pro

(GP), poly- Gly-Arg (GR), poly-Pro-Ala (PA), and poly-Pro-Arg (PR). DPRs have been identified to localise in neurons, forming TDP-43-negative, p62-positive pathological inclusions in *C9ORF72* patients (Al-Sarraj *et al.*, 2011; Murray *et al.*, 2011; Renton *et al.*, 2011; Gijselinck *et al.*, 2012; Ash *et al.*, 2013; Mann *et al.*, 2013; Mori, Weng, *et al.*, 2013). Functional studies in yeast, neurons in culture and *Drosophila* have shown that poly-GR and PR are highly toxic, whereas the other dipeptides seem to have a milder effect (Kwon *et al.*, 2014; Mizielinska *et al.*, 2014; Wen *et al.*, 2014; Freibaum *et al.*, 2015; Jovičić *et al.*, 2015; Tao *et al.*, 2015; Yang *et al.*, 2015). It is important to keep in mind that these pathogenic mechanisms are not mutually exclusive and that they could coexist in order for the disease to manifest. Further research in this field is necessary in order to draw any conclusion on *C9ORF72* mechanism of action.

Several studies have focused on the development of a target specific treatment for C9ORF72 patients using antisense oligonucleotide (ASO) direct against GGGGCC RNA transcripts (Donnelly *et al.*, 2013; Sareen *et al.*, 2013; Jiang *et al.*, 2016). This is a promising approach that has been tested on iPS cellular model as well as primary fibroblasts and has shown some success, reducing RNA foci without affecting the level of total C9ORF72 mRNA (Donnelly *et al.*, 2013; Lagier-Tourenne *et al.*, 2013). However, further investigation in animal models and eventually through clinical trials is needed to determine whether these ASOs are well tolerated and have a beneficial therapeutic effect on disease progression in ALS.

1.4.2.2 Genes discovered through family-based studies

Family-based studies have successfully identified genes involved in the pathogenesis of ALS (Al-chalabi, Berg and Veldink, 2016). Most of these genes have been found through genetic linkage studies, a technique that uses statistical analyses to determine whether a set of genetic markers co-segregate with disease in one or more large pedigrees. These studies have their limitations because ALS is a rapidly fatal, late onset disease with incomplete penetrance, which means that DNA is rarely available from multiple affected individuals in large pedigrees that have the power to identify novel loci by linkage. The

genes that have been discovered through linkage studies date back to the 1990s and early 2000s, and the more advanced techniques of GWAS, WES and WGS are now used for gene discovery. Here I describe the genes that have been reported using family-based linkage studies in ALS.

1.4.2.2.1 Ubiquilin 2 (*UBQLN2*)

Ubiquilin 2 (*UBQLN2*) was identified to be associated with ALS in 2011, when five mutations were reported in unrelated families of patients with dominant X-linked ALS (Deng *et al.*, 2011). Three of these mutations did not always segregate with disease, suggesting that *UBQLN2* might present incomplete penetrance (Daoud and Rouleau, 2011). More than 10 mutations have been found in *UBQLN2* since then, representing ~1% of fALS and a very small percentage of sALS. These variants have been reported to cluster in the proline rich domain with some exceptions (Deng *et al.*, 2011; Daoud, Suhail, *et al.*, 2012; Synofzik *et al.*, 2012; Williams *et al.*, 2012; Gellera *et al.*, 2013). ALS patients harbouring mutations in *UBQLN2* present an early age of onset and disease duration of six months to five years, these characteristics are exacerbated in male patients (Deng *et al.*, 2011; Gellera *et al.*, 2013). Clinically *UBQLN2* cases show upper and lower motor neuron symptoms with a high incidence of dementia, making these mutations strongly associated with ALS-FTD (Deng *et al.*, 2011; Gellera *et al.*, 2013). Histopathological investigation of post mortem tissue showed UBQLN2 inclusions, which often colocalise with other protein involved in ALS pathology such as TDP-43, FUS and Optineurin (Deng *et al.*, 2011; Williams *et al.*, 2012). Interestingly, these inclusions were also found in ALS patients non carriers of *UBQLN2* variants and presenting ALS-dementia phenotype, suggesting a correlation between UBQLN2 pathology and ALS associated dementia (Daoud and Rouleau, 2011). UBQLN2 is involved in protein degradation through the Ubiquitin-Proteasome System (UPS) (Ko *et al.*, 2004) and in autophagy, a process through which ubiquitinated proteins and dysfunctional organelles are degraded in autophagosomes (Ajroud-Driss and Siddique, 2014). This suggests that UBQLN2 pathogenesis might involve impaired protein degradation, a hypothesis

supported by the identification of ALS associated variants in other autophagy related genes such as Optineurin (Maruyama *et al.*, 2010) and VCP (Johnson *et al.*, 2010).

1.4.2.2.2 *Optineurin* (*OPTN*)

OPTN was eported to be associated with ALS for the first time in 2010 (Maruyama *et al.*, 2010) by a Japanese group using homozygosity mapping. Mutations in this gene are now considered to account for ~3% of familial ALS and ~1% of sporadic ALS (Ling, Polymenidou and Cleveland, 2013). Since 2010 ~40 *OPTN* variants (missense and nonsense) have been reported (Maruyama *et al.*, 2010; Del Bo *et al.*, 2011; Iida *et al.*, 2012; Tümer *et al.*, 2012; Van Blitterswijk, Van Vught, *et al.*, 2012; Weishaupt *et al.*, 2013; Soong *et al.*, 2014; Li *et al.*, 2015; Özoğuz *et al.*, 2015; Fifita *et al.*, 2016; Goldstein *et al.*, 2016), with a higher frequency in non-European populations, in particular the Japanese and Chinese. The frequency and penetrance of specific *OPTN* mutations varies with the ethnicity, hinting towards a genetic founder effect (Markovinovic *et al.*, 2017). *OPTN* variants have also been linked to primary open angle glaucoma (POAG) (Rezaie *et al.*, 2002; Minegishi *et al.*, 2016) and to Paget's disease of the bone and Chron's disease of the bowel (Albagha *et al.*, 2010; Obaid *et al.*, 2015; Smith *et al.*, 2015). The histopathology of OPTN-linked ALS is not well characterised. Some evidence of OPTN inclusions, positive for TDP-43 and p62, have been previously observed (Ito *et al.*, 2011; Kamada *et al.*, 2014) however, they do not seem to be a feature of OPTN mutant pathology. Interestingly, wild type OPTN has been found to colocalise with cytosolic inclusions in other neurodegenerative diseases such as Alzheimer's (AD), Parkinson's (PD), Huntington's (HD), Pick's and Creutzfeldt-Jakob's disease, and sporadic forms of ALS (Maruyama *et al.*, 2010; Hortobágyi *et al.*, 2011; Osawa *et al.*, 2011; Ling, Polymenidou and Cleveland, 2013). This can be explained by the ability of OPTN to participate in recruiting the autophagy machinery. This protein, in fact, is involved in a wide range of pathways from the negative regulation of NF-kB (Zhu *et al.*, 2007) to the autophagy receptor function, particularly relevant for ALS and neurodegenerative diseases in general, as impaired autophagy has been previously

suggested as a possible pathogenic mechanism for these disorders (Wild *et al.*, 2011; Wong and Holzbaur, 2014; Heo *et al.*, 2015; Lazarou *et al.*, 2015). OPTN has also been reported to be involved in the autophagosome maturation process (Tumbarello *et al.*, 2015), through interaction with TBK1 (another ALS-associated protein), in the defence against pathogens (Pourcelot *et al.*, 2016) and in mitophagy, a mitochondria specific type of autophagy (Matsumoto *et al.*, 2015). Although involved in numerous pathways, it is still not clear how OPTN mutations might cause ALS and further research is necessary in order to elucidate the precise role of OPTN in disease pathogenesis.

1.4.2.2.3 *ALS2*

ALS2 variants have been identified in patients that present a rare form of juvenile onset, slowly progressive ALS (ALS2) characterised by autosomal recessive transmission (Hadano *et al.*, 2001). Most of the *ALS2* variants found to date are predicted to produce a truncated form of the protein, with a disease phenotype that seems to be directly proportional to how severe the truncation is (Hadano *et al.*, 2001; Yang *et al.*, 2001). Given that the disease becomes evident only in the recessive state, where mutations are commonly homozygous, ALS2 is clearly due to a 'loss of function' mechanism (Pasinelli and Brown, 2006). *ALS2* codes for a ubiquitously expressed 184 kDa protein, alsin, which is particularly abundant in neurons where it localises in the endosomal membrane and appears to be involved in endosomal trafficking (Otomo *et al.*, 2003). Mice lacking in alsin do not show any signs of neuron degenerations, however, they do present high oxidative stress (Cai *et al.*, 2005) as well as age-related neurological impairment and compromised vesicle and endosomal trafficking (Hadano *et al.*, 2001).

1.4.2.2.4 Vesicle-Associated Membrane Protein (*VAMP*)/synatobrevin-associated protein B (*VAPB*)

A large Brazilian pedigree affected by atypical ALS as well as SMA was found to carry a missense mutation (p.P56S) in *VAPB* (Nishimura *et al.*, 2004). Later, another missense mutation was identified in a cohort of 107 fALS (Chen *et al.*, 2010). However, no further

VAPB variants have been found since then. Expression of these variants in mammalian cells causes ER fragmentation and cytosolic aggregates, which appear to sequester the wild type form of *VAPB* encoded protein (Nishimura *et al.*, 2004; Kanekura *et al.*, 2006; Teuling *et al.*, 2007; Chen *et al.*, 2010). Transgenic mice expressing p.P56S *VAPB* variant in the nervous system do not show reduced survival, however, they do display cytosolic aggregation of TDP-43, SQSTM1 and ubiquitin at 18 months of age (Tudor *et al.*, 2010; Qiu *et al.*, 2013).

1.4.2.2.5 Senataxin (*SETX*)

Heterozygous missense mutations in *SETX* are associated with a juvenile onset, slow progression form of ALS (ALS4), which mostly affects LMN but is not fatal (Chen *et al.*, 2004; Zhao *et al.*, 2009; Avemaria *et al.*, 2011). Homozygous or compound heterozygous mutations in this gene have also been observed in an autosomal recessive form of ataxia with oculomotor apraxia (Moreira *et al.*, 2004; Duquette *et al.*, 2005; Anheim *et al.*, 2009). *SETX* encodes for DNA/RNA helicase, senataxin, which is involved in DNA repair and RNA synthesis (Chen *et al.*, 2004), suggesting once again that RNA metabolism could play an important role in ALS pathogenesis.

1.4.2.2.6 hnRNPs

The heterogeneous nuclear ribonucleoprotein (hnRNP) family is a category of proteins that binds to newly synthesised RNA and participate in its transport and maturation (Piñol-Roma *et al.*, 1988; Dreyfuss *et al.*, 1993; Jean-Philippe, Paz and Caputi, 2013). hnRNPs, together with other important ALS-linked proteins such as FUS and TDP-43, bear a prion-like domain (PrLD), which, when mutated, makes these proteins particularly prone to aggregation. Only three variants for hnRNPA1 and one for hnRNPA2/B1 have been found in patients with ALS or multisystem proteinopathy (MSP), a group of neurodegenerative diseases that includes ALS (Kim *et al.*, 2013; Q. Liu *et al.*, 2016). Interestingly, both hnRNPA1 and hnRNPA2/B1 have been found to be expressed at lower levels in the CNS of Alzheimer's patients (Berson *et al.*, 2012), however the

mechanism of pathogenicity of these protein variants is, to date, unclear. Their aggregation prone properties seem to suggest that misfolding and fibrilisation might be involved in causing the disease, leading to a toxic gain of function hypothesis. However, a few studies have also suggested that their pathogenicity might be linked to a 'loss of function' (Kapeli, Martinez and Yeo, 2017).

1.4.2.3 Genes discovered through a candidate gene approach

The candidate gene approach uses what is known about a specific gene (i.e. biological function, association with diseases etc.) to associate it to the disease of interest. This technique has been fairly successful although severely criticised. Studies that found candidate genes through this approach, in fact, have been particularly difficult to replicate (Zhu and Zhao, 2007). Furthermore, it is worth noticing that a prior association of the gene with a similar disorder or with a relevant molecular mechanism is necessary in order to perform this gene-discovery technique. This method, therefore, heavily relies on prior knowledge of the gene's biological, physiological or functional role, which might be insufficient to make a correct prediction. It is believed that this 'hypothesis driven' technique might be less effective than an 'anonymous' technique such as WES or GWAS, which do not depend on the ability to make a biologically plausible hypothesis (Tabor, Risch and Myers, 2002). Nonetheless, a few ALS-linked genes have been found using this method and they are listed below.

1.4.2.3.1 SQSTM1/p62

SQSTM1 encodes for a 440 amino acid protein Sequestosome 1, also named p62. This protein has been shown to colocalise with TDP-43, FUS and ubiquilin2 inclusions in ALS as well as other neurodegenerative diseases (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009; Al-Sarraj *et al.*, 2011; Marangi and Traynor, 2014; Bury *et al.*, 2016). A candidate gene approach was, therefore, applied and rare mutations in *SQSTM1* were found in 1-2% of fALS and up to 4% of sALS (Fecto *et al.*, 2011; Rubino *et al.*, 2012; Teyssou *et al.*, 2013; Chen *et al.*, 2014; Kwok, Morris and de Belleroche, 2014). *SQSTM1* variants were

also previously found in patients affected by Paget's disease of the bone (Laurin *et al.*, 2002). The presence of mutations on *SQSTM1* was observed to be accompanied by TDP-43 pathology in ALS (Teyssou *et al.*, 2013) and p62 inclusions in other neurodegenerative diseases (Brettschneider *et al.*, 2012). p62 acts as an autophagy adapter, targeting polyubiquitinated proteins that are then processed by the proteasome or through other autophagy pathways (Moscat and Diaz-Meco, 2012). Although the pathogenic mechanism of these variants is still not clear, the strong association of p62 with autophagy seems to be the more plausible explanation to mutant *SQSTM1* pathogenesis (Ling, Polymenidou and Cleveland, 2013).

1.4.2.3.2 Ataxin 2 (*ATXN2*)

Ataxin 2 (*ATXN2*) was reported to bear a CAG expansion corresponding to a 34 amino acid long poly-glutamine repeat in patients affected by Spinocerebellar ataxia type 2 (SCA2) (Elden *et al.*, 2010). Some of these patients reported some motor neuron degeneration and this led to the formulation of the hypothesis that *ATX2* could have been involved in ALS. Many reports have now shown a correlation between mutations in this gene and ALS. In particular, 27-33 repeats have been associated with an ALS phenotype, 34 repeats with SCA2 manifestations and <27 with no clinical phenotype (Elden *et al.*, 2010; Chen *et al.*, 2011; Daoud *et al.*, 2011; Ross *et al.*, 2011; Van Damme *et al.*, 2011; Yu *et al.*, 2011; Gispert *et al.*, 2012; Van Langenhove *et al.*, 2012). This expansion accounts for 5% of all ALS cases (Morgan and Orrell, 2016). ATXN2 was found to aggregate and colocalise with TDP-43 in inclusions localised in the spinal cord of ALS patients (Elden *et al.*, 2010). Interestingly, a recent study showed that *ATXN2* knockout or knock-down using ASOs to ATXN2 mRNA can significantly increase TDP-43 transgenic mice survival (Becker *et al.*, 2017).

1.4.2.3.3 Heavy Neurofilament Subunit (*NEFH*)

Insertion and deletion variants of *NEFH* have been reported in ALS patients by three independent studies (Figlewicz *et al.*, 1994; Tomkins *et al.*, 1998; Al-Chalabi *et al.*,

1999). These variants were mainly found in sALS and did not show any segregation, which might be due to a low penetrance of *NEFH* variants (Al-Chalabi *et al.*, 2012). No further *NEFH* variants have been associated with ALS patients since the late 1990's. However, mutations in *NEFH* have recently been identified as a cause of Charcot-Marie-Toooth Disease (CMT) in four families (Rebelo *et al.*, 2016; Jacquier *et al.*, 2017). The mechanism of pathogenicity of NEFH is not clear, however, degenerating lower motor neurons of ALS patients often present neurofilamentous inclusions. Furthermore, NEFH loss of function might cause impaired axonal transport (Al-Chalabi *et al.*, 2012; Renton, Chiò and Traynor, 2013).

1.4.2.3.4 *Charged multivesicular body protein 2B* (*CHMP2B*)

Rarely identified in ALS cases (~1% fALS), variants in this gene were initially reported in a large Danish pedigree with FTD (Skibinski *et al.*, 2005; Parkinson *et al.*, 2006). In ALS, variants of this gene, although rare, seem to be correlated with a form of the disease that prevalently affects lower motor neurons with variable age of onset (Cox *et al.*, 2010). CHMP2B is an important element of the enodosomal sorting complexes and a few studies have shown that CHMP2B mutants might impair the endosome-lysosome mediated autophagy (van der Zee *et al.*, 2008; Urwin, Authier, *et al.*, 2010; Han *et al.*, 2012). However, the pathogenic mechanism of *CHMP2B* variants are still unclear.

1.4.2.3.5 FIG4

Mutations in *FIG4* have been initially found to cause Charcot Marie Tooth (CMT) disease (Chow *et al.*, 2007). One of the members of the families involved in the CMT study presented motor symptoms compatible with ALS manifestation. For this reason, Chow and colleagues decided to screen ~500 ALS patients variants in this gene and found 9 variants (Chow *et al.*, 2009). Since then no more *FIG4* variants have been found in ALS cohorts. *FIG4* encodes for a lipid phosphatase involved in regulating the amount of phosphatidyl-inositol-3,5-biphosphate (PI(3,5)P2). *FIG4* null mice have weakly expressed PI(3,5)P2, resulting in disrupted autophagy in neurons and astrocytes, caused

by impaired formation and recycling of autolysosomes (Ling, Polymenidou and Cleveland, 2013). FIG4 positive inclusions were observed in the CNS of patients affected by Parkinson's, Pick's disease and Lewis body dementia (Kon *et al.*, 2014).

1.4.2.3.6 *TAF15* and *EWSR1*

After the discovery of TDP-43 and FUS involvement in ALS, the theory that RNA binding proteins might be linked to the disease pathogenesis became a popular belief. For this reason a few studies screened TATA-binding protein associated factor 15 (*TAF15*) and Ewing Sarcoma RNA-Binding Protein 1 (*EWSR1*) for variants in ALS patients (Couthouis et al. 2011a, 2012; Ticozzi et al. 2011). These two genes encode for two proteins that belong, together with FUS, to the FET family and are, therefore, structurally and functionally very similar to FUS and TDP-43 (Zufiría *et al.*, 2016). To date, *TAF15* variants have been found in six families, while three *EWSR1* variants have been reported in sporadic cases (Couthouis et al. 2011b, 2012; Ticozzi et al. 2011). Due to their low complexity domain these proteins are both prone to aggregation and they have been found to mislocalise and aggregate in the cytosol of motor neurons of ALS patients (Couthouis *et al.*, 2011, 2012).

1.4.2.3.7 Angiogenin (*ANG*)

Chosen as a candidate gene because in linkage disequilibrium with *APEX1*, previously associated with ALS (Hayward *et al.*, 1999), *ANG* gene encodes for another protein involved in RNA homeostasis (Ling, Polymenidou and Cleveland, 2013). To date, 17 missense mutations have been identified in ALS cases (Chiò et al. 2012; Greenway et al. 2004, 2006; Millecamps et al. 2010). However, only one of these mutations, occurring at higher frequency compared to the others, has been shown to segregate with ALS in a Dutch kindred (Pan *et al.*, 2015). The involvement of *ANG* in ALS remains controversial as some of the supposedly pathogenic variants have been identified in healthy controls. Furthermore, some of the patients bearing mutations in *ANG* have also been found to

carry mutation in other ALS genes so it may be acting in conjunction as an oligogenic risk gene (Luigetti *et al.*, 2011; Lattante *et al.*, 2012).

1.4.2.4 Genes discovered through whole exome sequencing

Whole Exome Sequencing (WES) allows the recording of all the protein coding region of all known human genes (exome, roughly 2% of the whole genome). This powerful technique has been successfully applied in ALS gene hunting for the past few years and has identified a large number of genes involved in ALS pathogenesis, which are listed below. Whole exome sequencing is particularly useful in diseases, like ALS, that are late in onset, rapidly fatal and have low penetrance, which make traditional linkage studies challenging. One limitation of WES is that it fails to identify variants that map to non-coding regions of the genome such as the intronic G_4C_2 repeat in *C9ORF72*. However, the majority of ALS gene mutations are in exons, making WES an ideal approach. Whole genome sequencing is now the preferred methodology but challenges remain in terms of manipulating such dataset, therefore prioritising exome analysis is, at the moment, the most efficient strategy for gene hunting in ALS.

1.4.2.4.1 Vasolin Containing Protein (*VCP*)

Vasolin Containing protein (*VCP*) was the first ALS-linked gene to be identified through a WES study (Johnson *et al.*, 2010). This gene had already been associated with a form of FTD accompanied by Paget's disease of bone and inclusion body myopathy (IBMPFD) (Watts *et al.*, 2007). In some cases, overlapping between the two conditions exists as the *VCP* variants discovered in IBMPFD are also present in ALS cases. *VCP* mutations account for 1-2% of fALS, these patients show classic ALS phenotype with some evidence of cognitive impairment (Johnson *et al.*, 2010). This gene encodes for the VCP protein (also called p97) that is involved in major autophagy pathways. Knock down of VCP in mammalian cells causes accumulation of immature autophagosomes, suggesting that this protein plays an important role in autophagy and that its dysfunction may lead to TDP-43 aggregation (Ju *et al.*, 2009; Tresse *et al.*, 2010). More generally, VCP is

involved in the degradation and recycling of ubiquitinated proteins, and has a similar role as other genes linked to ALS (i.e. SQSTM1, UBQLN2). Mutant VCP has been associated with mitochondria uncoupling causing a decrease of ATP production, which might explain the effect in different tissues (Bartolome *et al.*, 2013).

1.4.2.4.2 Spatacsin (*SPG11*)

Mutations in *SPG11* are the most frequent cause of hereditary spastic paraplegia associated with a thin corpus callosum (Stevanin *et al.*, 2007). Using exome sequencing, *SPG11* variants were later linked to ALS in cases presenting a recessive juvenile form of the disease. These patients presented mean age of onset of ~16 years old and slowly progressive phenotype with a mean survival of 34 years (Orlacchio *et al.*, 2010; Daoud, Zhou, *et al.*, 2012). *SPG11* encodes for a 2443 amino acid protein, spatacsin. Although its physiological function is unknown, this protein has been shown to be required for the correct development of spinal motor neurons in zebrafish (Martin *et al.*, 2012).

1.4.2.4.3 Profilin 1 (*PFN1*)

In 2012 another WES study reported *PFN1* mutations in families with a form of autosomal dominant familial ALS (Wu *et al.*, 2012). This study suggested a frequency of 1-2% *PFN1* variants in fALS, however, many studies have failed to identify mutations in this gene at a similar frequency (Chen *et al.*, 2013; Daoud *et al.*, 2013; Dillen *et al.*, 2013; Ingre *et al.*, 2013; Lattante *et al.*, 2013; Tiloca *et al.*, 2013; van Blitterswijk *et al.*, 2013; Yang *et al.*, 2013; Zou *et al.*, 2013). *PFN1* encodes for profilin 1, a protein that plays an important role in the regulation of actin polymerisation (Witke, 2004). When overexpressed in immortalised cell lines or in mouse primary motor neurons, mutant profilin 1, but not its wild type form, clusters in ubiquitinated aggregates (Wu *et al.*, 2012), suggesting that mutant profilin 1 drives disease pathogenesis through a toxic gain of function mechanism. Overexpression of human mutant profilin 1 in mice causes progressive paralysis, motor neuron loss and premature death (Yang *et al.*, 2016). Mutant PFN shows reduced ability to bind to actin *in vitro* which implies that a loss of

function disease mechanism may also exist (Wu *et al.*, 2012). Furthermore, the growth defect phenotype induced by the deletion of PFN1 orthologue in *C. Elegans* is rescued by the expression of human wild type but not mutant PFN1 (Figley *et al.*, 2014).

1.4.2.4.4 Matrin 3 (*MATR3*)

Mutations in *MATR3* were found to be implicated in ALS through exome sequencing of four large families of European ancestry and an additional 108 fALS patients (Johnson et al. 2014). To date, 15 *MATR3* variants have been identified accounting for ~0.8% of ALS cases (Fifita et al. 2014; Johnson et al. 2014; Leblond et al. 2016; Lin et al. 2015; Marangi et al. 2017; Millecamps et al. 2014; Origone et al. 2015; Xu et al. 2016). ALS patients bearing mutations in *MATR3* commonly present with bulbar dysfunction and a slowly progressive phenotype (≥15 years) (Johnson et al. 2014; Marangi et al. 2017; Origone et al. 2015). Mutations in *MATR3* have previously been associated with autosomal dominant, distal, asymmetrical myopathy with vocal cord paralysis (Feit *et al.*, 1998; Senderek *et al.*, 2009). *MATR3* encodes for a 125 kDa nuclear matrix protein, Matrin 3 (MATR3), which binds DNA and RNA and has been shown to interact with TDP-43 in an RNA dependent way (Johnson et al. 2014). p.S85C mutant showed increased interaction with TDP-43 compared to MATR3 wild type and other mutants. Furthermore, patients carrying p.S85C show MATR3 and TDP-43 positive inclusions in skeletal muscle tissue (Johnson et al. 2014).

1.4.2.4.5 Tubulin Alpha 4a (*TUBA4A*)

An association between TUBA4A and ALS was found through a burden analysis study of rare variants in an exome sequencing in familial ALS cases (Smith *et al.*, 2014). To date more than 10 variants have been linked to ALS with a low prevalence of FTD (Smith *et al.*, 2014; Pensato *et al.*, 2015). However, no *TUBA4A* variants have been found in a large Spanish cohort of FTD patients (Dols-Icardo, Iborra, *et al.*, 2015). Clinically patients carrying *TUBA4A* variants present mainly spinal onset with UMN and LMN features (White and Sreedharan, 2016). *TUBA4A* encodes for Tubulin Alpha 4a, a protein

involved in microtubule cytoskeleton formation. It has been shown that some ALS-associated TUBA4A variants aggregate and fail to be incorporated in microtubules in transfected cell line and primary motor neurons and compromise their acetylation (Smith *et al.*, 2014; Perrone *et al.*, 2017). This suggests that heterozygous mutant *TUBA4A* might have a dominant negative effect and cause the disease by disrupting transport leading to TDP-43 aggregation.

1.4.2.4.6 Coiled-coil-helix-coiled-coil-helix domain containing 10 (*CHCHD10*)

Mutations in *CHCHD10* were first reported in a large French family affected by ALS, FTD, mitochondrial myopathy and cerebellar ataxia (Bannwarth *et al.*, 2014). The same study showed that missense mutations in *CHCHD10* cause mitochondria fragmentation and respiratory chain deficiency. Many *CHCHD10* variants have been identified in ALS patients since then (Chaussenot et al. 2014; Chiò et al. 2015; Dols-Icardo et al. 2015; Johnson et al. 2014; Pasanen et al. 2016; Penttilä et al. 2016; Zhang et al. 2015) accounting for ~1% of ALS cases (Perry, Shin and Tainer, 2016). However, some of the disease associated variants have also been often found to be present in controls (Abdelkarim et al. 2016; Dols-Icardo et al. 2015; Marroquin et al. 2016; Wong et al. 2015). *CHCD10* encodes for a protein of unknown function. This has been found to be located in mitochondria intermembrane space where it is believed to play a role in oxidative phosphorylation, resulting in mitochondrial abnormalities when mutated (Bannwarth *et al.*, 2014; Chiò *et al.*, 2015).

1.4.2.4.7 Tank Binding Kinase 1 (*TBK1*)

Over 100 heterozygous *TBK1* variants have been identified in ALS or FTD patients in only two years from the discovery of the implication of this gene in these pathologies. Mutations in *TBK1* account for 1.9% of all ALS and FTD cases, making TBK1 the fourth most common genetic cause of ALS and FTD (Freischmidt *et al.*, 2016). Mutations in this gene were initially identified by two independent studies using exome sequencing (Cirulli *et al.*, 2015; Freischmidt *et al.*, 2015). Around 30 of the variants found to date are

nonsense mutations, resulting in a truncated and likely non-functional protein. These mutations have shown high penetrance (up to 75% penetrance in patients over 60 years old). However, these numbers are based on a very limited amount of clinical data. Further studies with higher number of patients are required in order to draw any conclusion on *TBK1* penetrance. The proposed hypothesis for *TBK1* pathogenicity is haploinsufficiency (Cirulli *et al.*, 2015) as most of the functional work on *TBK1* variants has shown a reduced amount or a loss of function of the protein (Freischmidt *et al.*, 2015; Gijselinck *et al.*, 2015; Kim *et al.*, 2016; Tsai *et al.*, 2016; Pozzi *et al.*, 2017; van der Zee *et al.*, 2017). *TBK1* encodes for a multifunctional kinase, Tank Binding Kinase 1 (TBK1), which is involved in many pathways such as autophagy, response against pathogens, inflammation and cell proliferation (Helgason, Phung and Dueber, 2013). An increased *TBK1* copy number has been previously associated with another neurodegenerative disease: primary open angle glaucoma (POAG) (Ritch *et al.*, 2014; Awadalla *et al.*, 2015; Cai *et al.*, 2017). Furthermore, heterozygous TBK1 loss of function has been associated with herpes simplex encephalitis in childhood, probably linked to a diminished toll like receptor 3 (TLR3) mediated immunity activity (Herman *et al.*, 2012).

TBK1 mechanism of pathogenicity in ALS is, to date, unclear however its pathology is similar to sporadic ALS with TDP-43 cytoplasmic inclusions. TBK1 is a multifunctional protein involved in many different pathways but the leading hypothesis is that mutations in TBK1 might cause defective autophagy. This pathway has already been extensively linked to ALS and many disease-associated genes are implicated in this mechanism (i.e. *UBQLN2*, *SQSTM1*, *OPTN*). TBK1 has been shown to phosphorylate autophagy receptors such as p62 and OPTN (Kiriyama and Nochi, 2015), promoting their ability to bind to LC3-II and ubiquitinated proteins (Heo *et al.*, 2015). More specifically, this mechanism has been observed in mitophagy, degradation of dysfunctional mitochondria through autophagy. In this pathway Parkin, OPTN and Nuclear Dot Protein 52 (NDP52) are involved in the activation of TBK1 (Heo *et al.*, 2015) that, in turn, phosphorylates p62, NDP52 and OPTN, enhancing their ability to bind defective mitochondria (Lazarou *et al.*, 2015). This suggests the existence of a positive feedback between OPTN, NDP52 and

TBK1 (Heo *et al.*, 2015; Lazarou *et al.*, 2015). A few functional studies have shown that some of the TBK1 variants found in ALS patients show defective binding of OPTN and/or lack of TBK1 kinase activity (Freischmidt *et al.*, 2015; Gijselinck *et al.*, 2015; Pozzi *et al.*, 2017). Furthermore, a recent study shows that a TBK1 variant, mapping in the domain responsible for OPTN binding, does not co-localise with mitochondria as the wild type form of TBK1 does, implicating a defective mitophagy process (Richter *et al.*, 2016). Neuropathology observed in a reduced number of ALS, FTD and ALS/FTD patients harbouring mutations in TBK1, has reported TDP-43 and p62 positive inclusions in various brain regions (Freischmidt *et al.*, 2015; Pottier *et al.*, 2015; Van Mossevelde *et al.*, 2016). These studies seem to support the autophagy/mitophagy theory, although no definite conclusion can be made, as more extensive studies are needed in order to clarify whether TBK1-mediated autophagy is actually involved in the pathogenesis of ALS and FTD.

TBK1 is also involved in the defence against pathogens (Kawai and Akira 2007). Functional work on some TBK1 variants found in patients affected by ALS or ALS/FTD, has shown reduced or null ability to activate Interferon β (IFNβ) (Freischmidt *et al.*, 2015; Gijselinck *et al.*, 2015). *In vitro* and *in vivo* studies of the role of type I Interferons (IFNs) in ALS has shown a pleiotropic effect of this class of gene in neurone survival (van Boxel-Dezaire, Rani and Stark, 2006; Hofer *et al.*, 2010; Wang, Yang and Zhang, 2011). Therefore, a reduced ability of TBK1 to activate IFNβ might also play a role in ALS and FTD pathogenesis.

1.4.2.4.8 Annexin A11 (*ANXA11*)

In a recent exome sequencing study *Annexin A11* (*ANXA11*) mutations were associated with ALS as six mutations were identified in familial (1%) and sporadic (1.7%) ALS cases. Four of these variants were functionally investigated and showed defective binding of Calcyclin, which caused cytosolic aggregation of *ANXA11* encoded protein, Annexin A11 (ANXA11). This was observed in transfected cells and striking Annexin A11

inclusions were identified in the spinal motor neurons and neocortex of a patient carrying the most frequent p.D40G mutation (Smith *et al.*, 2017).

1.4.2.4.9 NIMA Related Kinase 1 (*NEK1*)

NIMA Related Kinase 1 (*NEK1*) mutations were described as strongly associated with ALS in an exome sequencing study of ~2900 patients and ~6400 controls (Cirulli *et al.*, 2015). This result was later confirmed by two other independent studies and it is now identified as a risk variant in 3% of European and European-American ALS cases (Brenner *et al.*, 2016; Kenna *et al.*, 2016). *NEK1* encodes for a kinase, NEK1, that has been predicted to interact with ALS2 and VAPB (Cirulli *et al.*, 2015), two proteins previously associated with ALS. Although promising, this gene cannot be considered causative of ALS until functional studies and/or neuropathology shows biological evidence of its involvement in the disease pathogenesis.

1.4.2.5 Genes identified through genome wide association studies (GWAS)

GWAS allows the identification of single nucleotide polymorphism (SNP) variants that are enriched in a disease cohort compared to matched controls through association analysis of genome-wide SNP data. This technique has successfully identified many genes associated with ALS (He *et al.*, 2015). However, the biggest limitation for the use of GWAS in gene hunting is the sample size needed in order to successfully identify disease associated variants. GWAS studies, in fact, require sample size that are vast and difficult to achieve especially for low frequency diseases like ALS (Purcell, Cherny and Sham, 2003). SNP variants identified through GWAS cannot be considered to be directly involved in causing ALS until biological evidence of a functional effect implicates their role in the disease pathogenesis. Here I present the genes, identified by GWAS studies, which have shown some evidence of their involvement in ALS.

1.4.2.5.1 Elongation Protein 3 (*ELP3*)

SNPs in *ELP3* were associated with ALS in a GWAS study of three different populations, which also showed that mutations in this gene in *Drosophila* cause neurodegeneration (Simpson *et al.*, 2009). This gene encodes for a protein, Elongation Protein 3 (ELP3), involved in RNA processing (Al-Chalabi *et al.*, 2012), suggesting once again that RNA metabolism may play an important role in ALS pathogenesis. However, no *ELP3* variant has been found, to date, in any familial or apparently sporadic case of ALS (Zufiría et al. 2016).

1.4.2.5.2 *UNC13A*

SNPs in this gene were associated with ALS susceptibility through a GWAS study (van Es *et al.*, 2009) and subsequently shown to influence survival (Jones *et al.*, 2017), a result that was confirmed by later studies (Koppers, 2011; Lill *et al.*, 2011). *UNC13A* was also identified as a modifying factor for survival and disease progression rate (van Es *et al.*, 2009; Koppers, 2011). This gene encodes for a protein, UNC13A, involved in the regulation of neurotransmitter release (van Es *et al.*, 2009). UNC13A deficient mice show impaired glutamate signalling and morphological anomalies in spinal cord neurons and neuromuscular junctions (NMJ) (Varoqueaux *et al.*, 2005).

1.5 Pathology

In the 1860s Charcot observed a loss of anterior horn cells causing muscle wasting (amyotrophy) and scarring (sclerosis) in the lateral corticospinal tracts of the spinal cord of patients affected by motor neuron disease, and named the disorder after this key neuropathological feature (Charcot and Joffroy, 1869). Since then, great progress has been made in our understanding of ALS pathology and its main characteristics will be explained below.

1.5.1 Macroscopic Features

Post mortem analysis of ALS patient nervous tissue has revealed loss of motor neurons in the motor cortex, in the brain stem and in the anterior horns of the spinal cord. In these patients muscles targeted by degenerating neurons undergo wasting and atrophy (Taylor et al. 2016). When dementia occurs, loss of neurons in the frontal and/or temporal cortex is often observed (Abrahams *et al.*, 2005; Chang *et al.*, 2005; Murphy *et al.*, 2007). Autopsies of ALS patients have also revealed a loss of white matter, which appears most marked in the cortico-spinal tracts (Kiernan and Hudson, 1994; Kassubek *et al.*, 2005; Roccatagliata *et al.*, 2009).

1.5.2 Microscopic Features

The main pathological hallmark of ALS discovered to date is the presence of protein aggregates that are often ubquitinated and referred to as inclusions (Leigh *et al.*, 1988). These aggregates have occasionally been found, in more advanced stages of the disease, co-localising with eosinophilic inclusions called “Bunina Bodies” (Okamoto, Mizuno and Fujita, 2008), however, the biological significance of these remains unexplained. TDP-43 aggregates take the form of fine and coarse skeins, granular or globular inclusions in the cytoplasm of upper and lower motor neurons and may also be present in the frontal and temporal lobe neurons (Figure 1.3) (Arai *et al.*, 2006; Neumann *et al.*, 2006; Mori *et al.*, 2008). There are a few pathological hallmarks, briefly described in section 1.4.2, which are gene specific. For instance, patients that harbour the hexanucleotide expansion on *C9ORF72* display intranuclear and cytosolic RNA foci in the cerebellum and hippocampus, as well as p62 and ubiquitin positive but TDP-43 negative inclusions in the cerebellum and frontal and temporal lobes. These inclusions have a large stellate structure in the cytoplasm and a small round structure in the nucleus (Al-Sarraj *et al.*, 2011), which were subsequently shown to contain di peptide repeat proteins (Figure 1.3)(Taylor, Brown and Cleveland, 2016).

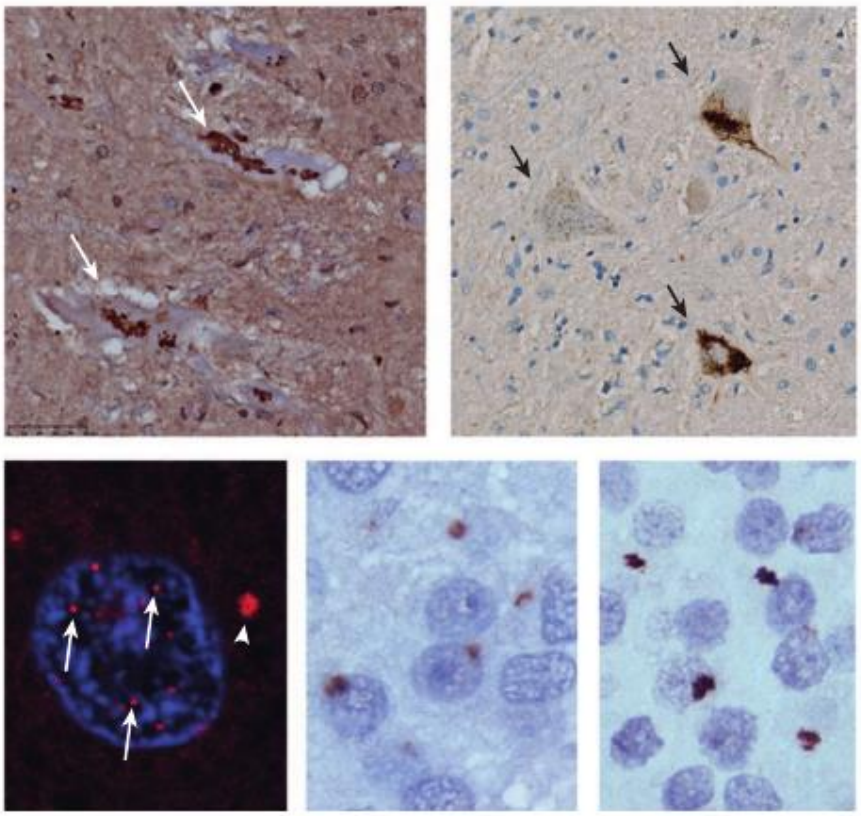


Figure 1.3 – Characteristic of ALS pathology. Images of tissue analysis showing (from left to right, top to bottom) SOD1 aggregates in spinal motor neurons, cytosolic TDP-43 in spinal motor neurons, RNA foci in the nucleus and cytosol of a C9 ALS-FTD cortical neuron, GA inclusions in the dentate nucleus of a C9ORF72 patient and GR inclusions in the dentate nucleus of a C9ORF72 patient (adapted from Taylor et al. 2016).

Another example of gene specific neuropathology is presented by SOD1 patients, who are negative for TDP-43 inclusions, but display SOD1 positive aggregates in motor neurons (Figure 1.3) as well as in surrounding cells such as glial cells (Bosco et al. 2010; Forsberg et al. 2010, 2011). Neuroglia has been shown to play an ambiguous role in ALS presenting both neuroprotective and toxic activity (described in section 1.6.8). These might be deleterious for neuron survival through a decline in the production of neurotrophic factors, or the release of neurotoxic agents and defective regulation of glutamate receptor homeostasis (Staats and Van Den Bosch, 2009).

1.6 Pathophysiology of ALS

The study of ALS genetics has enabled the creation of *in vivo* and *in vitro* models that have been used to shed light on the biological mechanisms behind ALS, illustrated in Figure 1.4. Although substantial progress has been made, the reason behind motor neuron degeneration is still not clear. ALS is a complex disease and it is believed that several mechanisms are able to contribute to its pathophysiology. Here I will present evidence for the major cellular mechanisms identified to date.

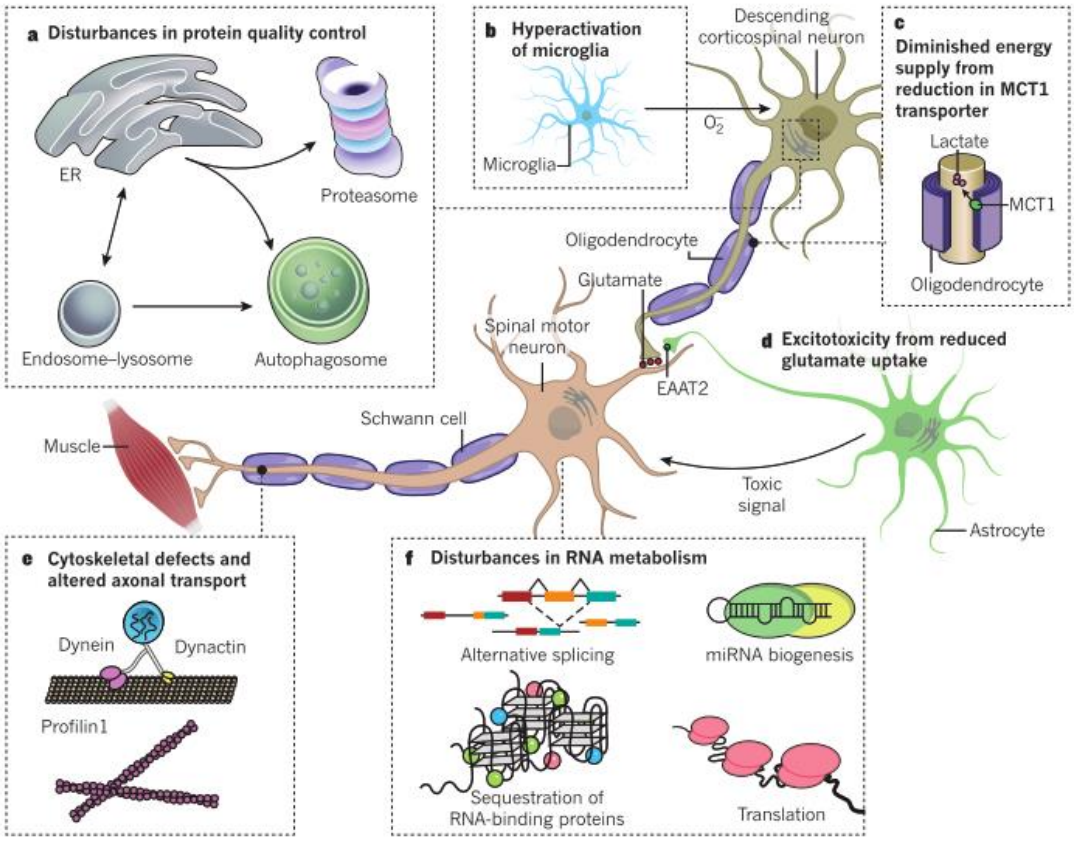


Figure 1.4 – Overview of ALS disease mechanism. (adapted from Taylor et al. 2016).

1.6.1 Protein aggregation

Protein aggregates are a hallmark of ALS and neurodegenerative disease in general (Lagier-Tourenne, Polymenidou and Cleveland, 2010). These inclusions are often found in the cytoplasm of spinal motor neurons and they are thought to overload protein homeostasis mechanisms, causing further stress to the cell (Saxena and Caroni, 2011;

Bendotti *et al.*, 2012). The major component of these aggregates is TDP-43, which accumulates in the cytosol, possibly seeded from stress granules and prevents its entry to the nucleus leading to nuclear depletion. Interestingly, the majority of ALS cases (95%) have TDP-43 positive inclusions and only 1% have *TARDBP* mutations, suggesting that genetic mutations are not essential to its aggregation (Coan and Mitchell, 2015). TDP-43 is often hyperphosphorylated, ubiquitinated and cleaved, which suggests that motor neurons harbouring these inclusions are somehow trying to remove them. Similarly, FUS has also been observed co-localising with stress granules and will form cytoplasmic inclusions in the motor neurons of ALS patients, although these patients always harbour genetic mutations in *FUS*.

As mentioned above (section 1.4.2.1.1), patients carrying mutations in the *SOD1* gene also show SOD1 protein inclusions that often present post-translational modifications. Mutant SOD1 result in an unstable protein that misfolds and accumulates in the cytoplasm to form SOD1-postive inclusions (Lindberg *et al.*, 2005). Interestingly, these inclusions have been found to also contain mutant as well as wild type and post-translationally modified wild type SOD1 (Bosco et al. 2010; Forsberg et al. 2010; Grad et al. 2014). These aggregates are thought to cause neuron death by toxic gain of function, altering the physiologic proteosomic function, obstructing axonal transport as well as other essential cellular activities (Bruijn *et al.*, 1997, 1998; Williamson and Cleveland, 1999).

Patients harbouring the pathological hexanucleotide G_4C_2 expansion in *C9ORF72* also display at least three different types of aggregates. As previously mentioned in section 1.4.2.1.4, the *C9ORF72* hexanucleoride expansion can be translated in five different dipeptide repeat protein through RAN translation: Poly-GP, Poly-GR and poly-GA, from the sense strand (Ash et al. 2013; Mori et al. 2013) and poly-PA, poly-GP and poly-PR, from the antisense strand (Gendron et al. 2013; Mori et al. 2013; Zu et al. 2013). These proteins aggregate in the hippocampus, frontal cortex, cerebellum, basal ganglia, motor cortex (Al-Sarraj *et al.*, 2011; Cooper-Knock *et al.*, 2012; Hsiung *et al.*, 2012; Mahoney *et*

al., 2012; Troakes *et al.*, 2012) and, in reduced amount, in the spinal cord (Gomez-Deza *et al.*, 2015; Schipper *et al.*, 2016) of ALS and FTD patients harbouring the *C9ORF72* G_4C_2 expansion long before symptoms onset (Proudfoot *et al.*, 2014; Baborie *et al.*, 2015). These inclusions show co-localisation with p62 and ubiquitin but not with TDP-43, however, *C9ORF72* FTD and ALS patients show TDP-43 pathology in the brain and spinal cord (Freibaum and Taylor, 2017).

Poly-GA is the most abundant DPR found in frontal and spinal cord p62/ubiquitin-positive aggregates in *C9ORF72* ALS and FTD patients (May *et al.*, 2014; Zhang *et al.*, 2014; Gomez-Deza *et al.*, 2015; Mackenzie *et al.*, 2015). These DPRs are the most aggregate prone and form amyloidogenic fibrils, which organise in parallel β-sheet structures that resemble the amyloid-beta peptide in Alzheimer's diseases (Chang *et al.*, 2016; Edbauer and Haass, 2016). Many *in vitro* studies have shown that this peptide has toxic features in neuronal cultures, which impair important cellular mechanisms such as endoplasmic reticulum homeostasis and proteasomal function (May *et al.*, 2014; Zhang *et al.*, 2014). These are thought to be caused by direct binding of poly-GA inclusions to important proteins involved in these processes (Freibaum and Taylor, 2017). However, the toxicity of these specific DPRs is controversial, a few studies, in fact, have shown that poly-GA DPR expression does not cause toxicity. Furthermore, even when toxic, poly-GA DPRs have been reported to have a far milder phenotype when compared to poly-GR and poly-PR DPRs expression (Mizielinska *et al.*, 2014; Wen *et al.*, 2014; Freibaum *et al.*, 2015).

Poly-PR and poly-GR are linear and highly charged arginine rich peptides, which form star shaped cytosolic inclusions that are highly toxic in cellular models (Kwon *et al.*, 2014; Wen *et al.*, 2014; Tao *et al.*, 2015; Lee *et al.*, 2016) as well as *Drosophila* models (Mizielinska *et al.*, 2014; Wen *et al.*, 2014; Freibaum *et al.*, 2015; Lee *et al.*, 2016). Expression of poly-PR and poly-GR have been shown to affect the formation and the physiologic role of membrane-less cellular structures such as nucleolus or stress granules (Lee *et al.*, 2016; Boeynaems *et al.*, 2017). Furthermore they also bind to proteins harbouring low complexity domains such as TDP-43 (Lin *et al.*, 2016).

Little or no toxicity has been shown for poly-GP and poly-PA. Poly GP inclusions have been observed with rare frequency in the brain or spinal cord of *C9ORF72* positive ALS and FTD patients (Gomez-Deza *et al.*, 2015; Mackenzie *et al.*, 2015) and neither of them has shown any toxicity when expressed in *Drosophila* neurons (Mizielinska *et al.*, 2014; Wen *et al.*, 2014; Freibaum *et al.*, 2015; Lee *et al.*, 2016).

The mechanism through which these different types of aggregates may cause cell death is still unknown. These inclusions might themselves be cytotoxic or might be caused by other pathway impairments. It has been proposed that defective protein degradation (i.e. autophagy and ubiquitin proteasome system) might be involved in the formation of these aggregates, this hypothesis will be discussed in the following section.

1.6.2 Protein degradation

Damaged organelles and defective proteins in cells are physiologically degraded by two interconnected physiological clearance mechanisms: the ubiquitin proteasome system (UPS) and autophagy. These processes have both been implicated in ALS by many genetic and pathological studies (Taylor et al. 2016). The UPS is usually devoted to the clearance of short-lived proteins and small protein complexes while the autophagy pathways degrade dysfunctional organelles and larger protein aggregates (Nixon, 2013). Autophagy is a multistep process that is tightly regulated, whereby a bi-layered vacuole called autophagosome is formed, which is devoted to the engulfment of intracellular components. This will later fuse with lysosomes, forming round structures called autophagolysosomes. The autophagosome carries on both sides of its membrane surface a protein called 1A/1B-light chain 3 (LC3), which plays an important role in autophagy as it is recognised by autophagy receptors such as OPTN and p62, which are mutated in some ALS patients. These receptors bind to the ubiquinated proteins/dysfunctional organelles and conduct them to the autophagosome in order to be degraded and recycled (Tanida, Ueno and Kominami, 2008; Kiriyama and Nochi, 2015; Oakes, Davies and Collins, 2017). Mutations in many proteins involved in the UPS and autophagy have been implicated in ALS including: *OPTN*, *UBQLN2*, *TBK1*, *VCP*,

SQSTM1, *CHMP2B*, *FIG4* and *VABP* have been described in ALS patients (see section 1.4.2), implicating defective proteostasis in the pathogenesis of ALS. When autophagy-related genes 5 or 7 (*Atg5* or *Atg7*), encoding proteins central to the autophagy process, are selectively downregulated in the CNS, mice develop sings of neurodegeneration (Hara *et al.*, 2006; Komatsu *et al.*, 2006). Furthermore, murine models of mutant SOD1 and apparently sporadic patients harbouring *SOD1* mutations have also shown an increase in the number of autophagosomes enclosed within their motor neurons (Morimoto *et al.*, 2007; Li, Zhang and Le, 2008; Hetz *et al.*, 2009; Tian *et al.*, 2011).

1.6.3 RNA metabolism and toxicity

Mutations in many RNA processing genes have been linked to ALS including: *TDP-43, FUS, MATR3, hnRNPs, C9ORF72, ATXN2, ELP3, SETX, TAF15* (described in section 1.4.2). These genes have all been associated with at least one aspect of RNA metabolism. Among these, mutations in *TDP-43* and *FUS* are the most frequent in ALS cases. These encode for two proteins that play a pivotal role in multiple steps regulating gene transcription, splicing, transport, translation as well as microRNA biogenesis (Ling, Polymenidou and Cleveland, 2013). A common feature of mutant TDP-43 and FUS proteins is their mislocalisation from the nucleus to the cytoplasm (Neumann *et al.*, 2006; Sreedharan *et al.*, 2008; Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). It is not yet clear whether their pathogenicity is due to a toxic gain of function, due to their aggregation in the cytoplasm, or by a loss of nuclear function or a combination of both (Peters, Ghasemi and Brown, 2015). Whatever the mechanism, years of investigation have clarified that there is a definite link between their alteration and ALS pathology. Many of these proteins are involved in RNA splicing where a loss of function of splicing activity could result in major disruption of protein synthesis. In line with these considerations, depletion of *TDP-43* in murine models has been reported to cause the alteration of splicing events in the brain, damaging the production of synaptic proteins (Polymenidou *et al.*, 2011). TDP-43 and FUS are also involved in the synthesis of small non-coding RNAs (micro RNAs, miRNAs), which are important in the regulation of gene expression. Knock out of

either *TDP-43* or *FUS* from *in vivo* and *in vitro* models has shown a downregulation of miRNAs expression, resulting in an altered RNA silencing activity (Z. Zhang *et al.*, 2013), which is consistent with what has been observed in the motor neurons of ALS patients (Valdez *et al.*, 2014). Interestingly, the hexanucleotide repeat identified in *C9ORF72* shows a different scenario. The *C9ORF72* gene is fully transcribed to RNA edited but it retains the G_4C_2 repeat containing intron that accumulates and aggregates to form intranuclear and occasionally cytoplasmic RNA foci, in neurons and glia in the brain and spinal cord (DeJesus-Hernandez *et al.*, 2011). Microsatellite expansions have been hypothesised to cause neuronal death by toxic gain of function in at least 22 neurological diseases. RNA foci, can sequester RNA binding proteins diseases, leading to the splicing defects illustrated above (La Spada and Taylor, 2010). Recent work has shown that these G_4C_2 repeats, when transcribed, can form a secondary structure called G quadruplexes, which makes the transcript particularly stable and potentially toxic, however there is no evidence that they directly bind TDP-43 (Taylor, 2014; Burguete *et al.*, 2015; Ishiguro *et al.*, 2016). Furthermore, G_4C_2 repeats have also shown to generate short RNA fragment that have similar functions to miRNAs, again possibly affecting the regulation of gene expression (Peters et al. 2015).

All these studies provide evidence that aberrant RNA metabolism plays an important role in the pathogenesis of ALS. Although considerable progress has been made in understanding some aspect of these mechanisms, further studies are needed to elucidate how RNA metabolism is involved in neuron death and whether it could represent a valuable therapeutic target.

1.6.4 Nucleocytoplasmic transport

The observation that cytosolic TDP-43 inclusions are present in the vast majority of ALS and ~45% of FTD cases (Ling, Polymenidou and Cleveland, 2013), led researchers to think that impairment of nuclear transport of TDP-43 might contribute to ALS aetiology (Winton *et al.*, 2008). Furthermore, FUS cases also present with FUS cytoplasmic inclusions and about half of the FUS ALS and FTD-linked mutations map in the nuclear

localisation signal (NLS) of FUS, affecting its translocation to the nucleus (Ling, Polymenidou and Cleveland, 2013). Interestingly, a strong correlation between NLS FUS mutations and the severity of the patient phenotype has been found (Bosco et al. 2010; Chiò et al. 2009; DeJesus-Hernandez et al. 2010; Dormann et al. 2010), suggesting that the disruption of nuclear transport exacerbates ALS and FTD pathology. In accordance with this, analysis of post mortem brain and spinal cord from ALS and FTD patients showed a lower expression and/or mislocalisation of proteins involved in nuclear import including karyopherin β1 and β2 (Nishimura *et al.*, 2010; Neumann *et al.*, 2012; Takeuchi *et al.*, 2013).

Moreover, four independent studies have recently shown, using different models expressing G_4C_2 repeats or DPR dipeptides, that a number of proteins involved in nucleocytoplasmic transport, including karyopherins, Ran-GTP cycle regulators (such as the nucleocytoplasmic transport regulators RanGAP1, which activates RAs-related Nuclear protein (RAN) and keeps it in the GTP-bound state, and RanGEF, nuclear promoter of the RAN bound GTP by GDP) and members of the Nuclear Pore Complex (NPC, including Nup50, Nup205 and Nup107, which are components of the nuclear basket), are affected in *C9ORF72 in vivo* and *in vitro* models (Freibaum *et al.*, 2015; Jovičić *et al.*, 2015; K. Zhang *et al.*, 2015; Boeynaems *et al.*, 2016). Interestingly, one of the genetic modifiers of toxicity identified by these studies is *GLE1,* a gene that codes for a nucleoporin protein, involved in nuclecytoplasmic trafficking, which has been recently identified in one familial ALS and two sporadic cases (Kaneb *et al.*, 2014). Impairment of nucleocytoplasmic transport is, therefore, considered one of the main ALS-FTD underlying mechanisms, especially in *C9ORF72* ALS cases (Kim and Taylor, 2017).

1.6.5 Cytoskeletal function

The cytoskeleton is a cellular structure made of filamentous proteins that gives cells mechanical support and its architecture defines the cells morphology and function. It is composed by three main classes of proteins (microtubules, actin filaments and intermediate filaments) and it is particularly important in neurons, where it supports the

axon and plays an important role in intracellular transport. Neurons and motor neurons in particular, whose axons can reach a length of one meter, heavily rely on their ability to transport molecules from the soma to the synaptic terminus (anterograde transport) and vice versa (retrograde transport) (Pasinelli and Brown, 2006). Microtubules form structures along the axon that can be used by proteins such as cargoes and motor proteins, which, hydrolysing ATP, can transport important cell components such as messenger RNA, organelles and ribosomes (De Vos *et al.*, 2008; Poppe *et al.*, 2014).

Very early on in ALS research, electron microscopy studies on patient material revealed axonal swellings enclosing aggregates of neurofilaments (Okamoto *et al.*, 1990; Sasaki and Maruyama, 1992). Since then, axonal transport dysfunction has been closely investigated in ALS pathology. Murine models of mutant SOD1 present impairment of axonal transport, which occurs in pre-symptomatic stages of the disease (Collard, Cote and Julien, 1995; Zhang *et al.*, 1997; Williamson and Cleveland, 1999; Kieran *et al.*, 2005; De vos *et al.*, 2007). Post mortem tissue and mouse models of ALS have also shown that motor neurons may retract their axons from their synaptic terminal very early on in the course of the disease, before any manifestation of neuronal death (Frey *et al.*, 2000; Fischer *et al.*, 2004). Furthermore, mutations in genes encoding cytoskeleton structure and axonal transport have been found in ALS patients, including: neurofilament heavy chain (NFH) (Figlewicz *et al.*, 1994; Tomkins *et al.*, 1998; Al-Chalabi *et al.*, 1999) and *Dynactin 1* (*DCTN1*) (Munch *et al.*, 2004; Stockmann *et al.*, 2013). This gene encodes for a protein, Dynactin 1, which plays a pivotal role in the retrograde axonal transport. In murine models, point mutations on *DCTN1* cause impaired vesicular transportation, motor neuron degeneration and early death (Puls *et al.*, 2003; Laird *et al.*, 2008).

A more recent example of the involvement of the cytoskeleton in ALS is the discovery of *TUBA4A* variants as a possible cause of motor neuron disease (Smith *et al.*, 2014). This gene encodes for a protein, Tubulin alpha-4A chain, which is one of the major components of microtubules. TUBA4A mutants have been shown to unsettle

microtubules assembly and stability, which will probably impair axonal transport in neurons (Smith *et al.*, 2014; Vos *et al.*, 2017). Other ALS linked genes involved in microtubules or axonal transport such as *PFN1* and have been described in section 1.4.2.

1.6.6 Mitochondrial dysfunction

Mitochondria are often described as the cell's lung. This complex organelle is involved in many crucial cellular processes such as: cell respiration and ATP synthesis, calcium homeostasis and apoptosis (Delettre *et al.*, 2000; Benard *et al.*, 2007). Mitochondria activity is particularly important in neurons as they lack an efficient glycolytic system. Furthermore, because of their polarised nature, neurons require mitochondria to be transported and placed in sites of intense metabolic demand (Sheng, 2014). Mitochondria dysfunction has been implicated in many neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's disease and ALS (Jiang *et al.*, 2015).

Evidence of mitochondrial dysfunction includes: reduced ATP production (Mattiazzi *et al.*, 2002; Le Masson, Przedborski and Abbott, 2014); loss of antioxidant activity and over production of reactive oxygen species (ROS, mainly associated with SOD1 variants) (Pitkanen and Robinson, 1996; Genova *et al.*, 2004; Adam-Vizi, 2005; Rizzardini *et al.*, 2005), as well as elevated Calcium (Ca^{2+}) levels in ALS patients (Siklós *et al.*, 1996) and animal models (Damiano *et al.*, 2006; Kim *et al.*, 2012; Parone *et al.*, 2013).

A closer look at mitochondria morphology has revealed that mitochondria are fragmented in mutant SOD1 models (Vande Velde *et al.*, 2011; Song *et al.*, 2013; Vinsant *et al.*, 2013; Magrané *et al.*, 2014) as well as in mutant TDP-43 *in vitro* and *in vivo* models (Y. Xu *et al.*, 2011; Y.-F. Xu *et al.*, 2011; Wang *et al.*, 2013; Magrané *et al.*, 2014). The fragmentation of mitochondria is the result of a tilted balance of mitochondria fission and fusion towards fission (Jiang *et al.*, 2015). This balance is important for the preservation of mitochondria function (Picard *et al.*, 2013) as fusion helps in the recycling of damaged

mitochondria and fission is involved in the generation of new mitochondria (Twig *et al.*, 2008).

Another important process involved in the recycling of damaged mitochondria is the mitophagy pathway. This is a mitochondria-specific type of autophagy and it is widely used by cells in order to maintain a healthy mitochondria population. Interestingly, several ALS-linked genes encode for proteins involved in this mitophagy including: *OPTN*, *TBK1*, *VCP*, *SQSTM1*. An example of this impairment, has been highlighted recently in the interaction between OPTN and TBK1. TBK1 can be activated by NDP52, Parkin or OPTN to function as a kinase and, in turn, phosphorylate NDP52, OPTN and p62. This activates these proteins, whose task is to bind LC3II and ubiquitin, functioning as autophagy receptors, which will flag damaged mitochondria and lead to their degradation by the autophagosome (Heo *et al.*, 2015; Lazarou *et al.*, 2015). Mutations in *OPTN*, *TBK1* or *SQSTM1* (p62) have been found in ALS patients and may disrupt mitophagy, resulting in an accumulation of damaged mitochondria, which are highly likely to contribute towards ALS by disrupting axonal transport (Oakes, Davies and Collins, 2017).

As mentioned above, mitochondria trafficking is particularly important for motor neurons, where, in physiological conditions, these organelles are concentrated at the synaptic terminals, which are sites of high metabolic demand (Jiang *et al.*, 2015). ALS patients, however, have been found to display a concentration of mitochondria in the soma of motor neurons within the spinal cord, which suggests a mitochondria trafficking impairment (Sasaki and Iwata, 2007) already highlighted as a possible disease mechanism in many neurodegenerative diseases (Guo *et al.*, 2005; Kieran *et al.*, 2005). This was confirmed by *in vitro* and *in vivo* studies of models expressing disease associated mutant TDP-43 (Igaz *et al.*, 2011; Janssens *et al.*, 2013; Wang *et al.*, 2013; Magrané *et al.*, 2014) and SOD1 (De vos *et al.*, 2007; Sotelo-Silveira *et al.*, 2009).

Another important mitochondria feature is their ability to associate with the endoplasmic reticulum (ER). Indeed, 5-20% of the total mitochondrial surface is in contact with the ER

through specific ER areas called Mitochondria Associated Membranes (MAMs) (Csordás *et al.*, 2006; Rowland and Voeltz, 2012; Helle *et al.*, 2013; van Vliet, Verfaillie and Agostinis, 2014). ER/mitochondria interaction is an important event which is thought to regulate a number of cellular processes including: Ca^{2+} exchange, intracellular trafficking and ER stress (Simmen *et al.*, 2005; MacAskill *et al.*, 2009; Rowland and Voeltz, 2012; Helle *et al.*, 2013; van Vliet, Verfaillie and Agostinis, 2014). MAMs have been found to be damaged in various neurodegenerative disorders including ALS and FTD (Area-Gomez *et al.*, 2012; De vos *et al.*, 2012; Ottolini *et al.*, 2013; Stoica *et al.*, 2014). An example of this is VAPB, which is an important MAM protein and has been found to be mutated in patients suffering from a severe form of ALS (Nishimura *et al.*, 2004)(section 1.4.2.2.4). Mutant VAPB is recruited to the MAMs in higher concentration than the wild type and causes major mitochondria calcium peaks by interacting with Protein Tyrosine Phosphatase-Interacting Protein 51 (PTPIP51), which tightens the interaction between the two organelles (De vos *et al.*, 2012). Furthermore, overexpression of TDP-43 causes a peak in Glycogen synthase kinase 3 beta (GSK3β) activation, which reduces the strength of ER-mitochondria interaction. Indeed, transgenic TDP43 mice show a lower amount of ER-mitochondria associations, a result that is confirmed by NSC34 cells overexpressing wild type and mutant TDP-43, which also show reduced calcium exchange (Stoica *et al.*, 2014). This highlights the importance of a balanced interaction between mitochondria and ER for neuronal health (Krols *et al.*, 2016).

How these impaired mitochondria dynamics contribute towards causing ALS remains to be determined, however, mitochondria abnormalities seem to occur early on in the disease, suggesting that these may play a key role in the pathogenesis of ALS (Jiang *et al.*, 2015).

1.6.7 Oxidative stress

Closely related to mitochondria dysfunction is oxidative stress, this occurs when the generation of reactive oxygen species (ROS), mainly through the mitochondria oxidative chain, exceeds their removal. Oxidative stress can cause structural damage to DNA and

other cell elements as well as alteration of epigenetic mechanism and RNA editing, resulting in possible variation of gene expression (Meaney and Szyf, 2005; Hunter and McEwen, 2013; Griffiths and Hunter, 2014; Reul, 2014). The involvement of oxidative stress in ALS was initially suggested by the analysis of human tissue from ALS patients, which showed evidence of oxidative insult to cell components such as proteins, lipids and DNA (Agar and Durham, 2003; Henkel *et al.*, 2004; Calingasan *et al.*, 2005; Kato *et al.*, 2005). This has been confirmed in cellular and murine models of mutant SOD1 (D'Amico *et al.*, 2013) and TDP-43 (Duan *et al.*, 2010; Deng, Gao and Jankovic, 2014; Jaronen, Goldsteins and Koistinaho, 2014), where oxidative stress may contribute to the formation of protein aggregates.

Oxidative stress was initially thought to be a stand-alone mechanism involved in the pathogenesis of ALS. It is now clear that this mechanism is deeply connected with mitochondria dysfunction and, probably, protein aggregation, which are, in turn, related to autophagy. This shows that ALS is a complex disorder and it is likely to be caused by more than one defective mechanism.

1.6.8 Neuroinflammation

Neuroinflammation, a specific type of inflammatory response that occurs in the CNS, is characterised by the infiltration of T lymphocytes, abundant synthesis of inflammatory cytokines and microglial and astrocyte activation in the nervous system. These events have been correlated with motor neuron loss in ALS, even in pre symptomatic stages, in animal and human tissue (Komine and Yamanaka, 2015). However, the implication of neuroinflammation in ALS pathogenesis is a double-edged sword, as immune cells have been shown to have both a damaging effect and a protective role for motor neurons (Liu and Wang, 2017).

Many studies have highlighted the importance of microglia in ALS, as the first line of immune defence within the CNS. However, sick motor neurons are able to release aggregated proteins, such as SOD1 or TDP43, which induces microglia to switch from

an M2 phenotype, which has a neuroprotective role, to an M1 phenotype, which stimulates the production of pro-inflammatory cytokines, causing neuronal damage (Appel *et al.*, 2011; Liao *et al.*, 2012; Roberts *et al.*, 2013; Zhao, Beers and Appel, 2013). Furthermore, murine models of mutant SOD1 (mSOD1) have shown that when the mSOD1 microglia was replaced with healthy glia, or when the expression of mSOD1 was selectively reduced in the mouse microglia, motor neuron degeneration was delayed and survival improved (Beers *et al.*, 2006; Boillee *et al.*, 2006).

Astrocytes are the most abundant glial cells in the CNS and they provide structural as well as functional support for neurons. Motor neuron toxicity has been shown to occur when astrocytes express mSOD1 *in vivo* and *in vitro*, with a toxic effect observed uniquely in motor neurons (Di Giorgio *et al.*, 2007, 2008; Nagai *et al.*, 2007).

T lymphocytes have shown to operate a protective action on motor neurons. The infiltration of $CD4^+$ T cells, immune cells devoted to the recruitment of white blood cells when in proximity of possibly damaging molecules, is observed early on in the disease (Bowerman *et al.*, 2013; Hooten *et al.*, 2015). These play a protective role interacting with M2 microglia and astrocytes, and have been found to be lowly expressed in the blood of ALS patients (Henkel *et al.*, 2013; Hooten *et al.*, 2015). Interestingly, genetic suppression of $CD4^+$ T cells increased disease progression (Beers *et al.*, 2008; Chiu *et al.*, 2008).

From this review, it is clear that disturbance of many different pathways can contribute to the pathogenesis of ALS, but their relative importance is debated and interaction is complex. Neuroinflammation has been acknowledged as one of the contributors to motor neuron degeneration, however, due to the disease heterogeneity and the ambiguous role of immune cells, further research is necessary in order to fully understand disease related neuroinflammatory mechanisms.

1.7 Therapy

ALS is an incurable disease and the only three drugs approved for the treatment of ALS by the Food and Drug Administration (FDA) in the US are: Riluzole, approved over twenty years ago, Nuedexta approved in 2010 and edaravone (Radicava), a free radical scavenger with neuroprotective properties (Sawada, 2017), approved in May 2017. Riluzole is the most commonly prescribed drug and it is believed to act by inhibiting glutamate release and reducing excitotoxicity, resulting in an extension of patient mean survival by three to six months (Bensimon, Lacomblez, and Meininger 1994; Lacomblez et al. 1996; Mitsumoto, Brooks, and Silani 2014).

The heterogeneity of ALS pathophysiology and genetics represents a great challenge in the development of a therapy that is effective for all patients. It is becoming increasingly clear that precision medicine will be the way forward in the treatment of this syndrome. In line with this, a few targeted treatments have been developed and are undergoing pre-clinical and clinical trials (Smith *et al.*, 2006; Kieran *et al.*, 2008; Rizvanov *et al.*, 2009; Williams *et al.*, 2009; Becker *et al.*, 2017). In 2013 a Phase I study reported that *SOD1* antisense oligonucleotides (ASOs) successfully completed a randomised phase one clinical trial on mutant *SOD1* fALS patients, which determined that it was safe (Miller *et al.*, 2013). A Multi-centered Phase II SOD1 ASOs study is now underway and a Phase I trial of ASOs to C9ORF72 G_4C_2 expansion mutations is due to start imminently. The complexity of ALS genetics and the modest numbers of mutant gene carriers, represents a significant obstacle in the development of a successful gene targeted therapy for sporadic ALS patients.

Another approach to treatment of ALS, and other neurodegenerative disorders, is stem cell transplantation, using either autologous or heterologous neuronal precursor or glial cells. Pre-clinical work has shown promising results in animal models (Silani *et al.*, 2004; Corti *et al.*, 2007, 2010; Giordano, Galderisi and Marino, 2007; Boucherie *et al.*, 2008; Suzuki *et al.*, 2008; Hwang *et al.*, 2009; Kim and de Vellis, 2009; Gu *et al.*, 2010; Wyatt

et al., 2011; Thomsen *et al.*, 2014) however the mechanism of benefit is not cell replacement, indeed very few if any of the transplanted cells differentiate into motor neurons. In 2010 the first clinical trial using neural stem cells (NSCs) and another clinical trial for the transplant of bone marrow derived mesenchymal stem cells (MSCs) on a mixed cohort of patients with ALS and multiple sclerosis (Karussis *et al.*, 2010) were approved in the US and Israel respectively. Both of these studies involved small patient numbers and have shown modest benefit. However, it is still too early to determine whether stem cell transplant will represent a useful therapy.

Despite over fifty years of clinical trials, ALS is still considered a terminal disease and management is largely palliative. Considerable advances in gene discovery have enabled the identification of novel molecular mechanisms involved in the pathophysiology of ALS. The study of genetic models has supplied novel therapeutic targets, providing hope that this will lead to the development of more effective therapies.

1.8 Study Aims

The review of the literature presented above highlights how our understanding of ALS has advanced dramatically with progress in genetic and pathophysiological studies but that translation into effective therapies is still awaited. Although substantial progress has been made, the genetic basis for ~40% of fALS and ~85% of sALS is yet to be determined. Furthermore, the disease mechanism for most of the genes that have been discovered is unclear. It is crucial to fill these gaps in our knowledge of ALS in order to design any effective treatment.

With this in mind, our team has carried out an extensive whole exome sequencing (WES) study of ~700 fALS index cases shared in a collaboration with other international researchers. WES has been proven to be a successful approach for gene discovery in ALS (section 1.4.2.4). Our team has identified several genes implicated in ALS (Smith *et al.*, 2014, 2017), however, we have also discovered novel variants in known ALS genes that have provided new insight into disease mechanisms. It is important to replicate and

further investigate recently discovered genes in order to corroborate early findings and extend our knowledge. Therefore, we decided to investigate two of these variant-enriched genes, *MATR3* and *TBK1*, in our cohort and in an additional ~200 apparently sporadic ALS cases. This characterisation, which will be discussed in the following chapters, intended to answer two main biological questions:

- What is the frequency and distribution of variants in *MATR3*/*TBK1* in our cohorts?
- How do these mutations affect physiology in cellular models and in patient material?

In addition to this, through an international collaboration, we had access to a Middle Eastern consanguineous kindred affected by ALS. WES and subsequent homozygosity mapping identified a novel nonsense *OPTN* mutation. The aim of this project was to functionally characterise this OPTN variant in patient material, in order to further investigate OPTN role in ALS.

The final aim of this PhD project was to shed light on the functional mechanisms that might be implicated in MATR3, TBK1 and OPTN related pathology, in the hope of improving our understanding of ALS pathophysiology and through these insights advance the development of more effective therapies.

Chapter 2 – Material and methods

2.1 DNA samples and Exome Sequences

2.1.1 Familial ALS (fALS) DNA samples

Analysis of fALS exomes was conducted via exome capture and next generation sequencing of DNA from the local Kings College London Motor Nerve clinic, the MNDA DNA Bank collection and DNA provided by collaboration from Frank Baas in Amsterdam, The Netherlands (n=12), Jacqueline de Belleroche in London, UK (n=18) and Kevin Talbot (n=4). All patients had a diagnosis of ALS according to the revised El Escorial criteria (Brooks *et al.*, 2000) and at least one affected relative, which classifies them as familial ALS cases (fALS). Furthermore, full patient consent was obtained for research. Raw exome sequence data from an additional 655 fALS patients was also provided by collaboration. The full cohort was, therefore, composed of 932 samples, primarily of European ancestry of which 684 were isolated index samples (first to be diagnosed and only representative sample of a family) and 248 were multiple affected relatives from 73 additional kindreds. These were originally from eleven countries, in descending order of group size: United States (283), United Kingdom (219), Italy (146), Spain (33), Germany (25), Israel (17), Ireland (17), Canada (9), Netherlands (4), Belgium (3) and New Zealand (1) (Table 2.1).

Patient Nationalities	Probands (Affected Relatives)	*Pathogenic Variants Identified
USA	283 (54)	15
UK	219 (65)	20
Italy	146 (25)	8
Spain	33 (11)	6
Germany	25 (2)	2
Israel	17 (4)	0
Ireland	17 (7)	3
Canada	9 (0)	1
Netherlands	4 (7)	2
Belgium	3 (0)	0
New Zealand	1 (0)	0
TOTAL	757 (932)	58

Table 2.1 – **Exome sequencing cohort.** Breakdown of patient nationalities, probands, affected relative and mutation identified per ethnic group. * Number of index cases harbouring likely pathogenic mutations in known (published before 2017) or novel ALS genes.

A small number of patients from the MNDA DNA Bank that had overlapping phenotypes such as Progressive Muscular Atrophy (PMA), Primary Lateral Sclerosis (PLS) and Progressive Bulbar Palsy (PBP) were also included in this study.

All fALS exome captured by King's were screened for mutations in known ALS genes (including *SOD1*, *C9orf72*, *TARDBP*, *FUS*, *PFN1*, *UBQLN2*, *OPTN*, *VCP*, and *ANG*). Mutant positive cases were excluded from novel variant analysis resulting in a final cohort of 699 probands. There has been increasing evidence in recent years of multiple oligogenic variants contributing to pathogenesis in some ALS patients (Marangi and Traynor, 2014), and so this approach may have prevented us from identifying novel variants in carriers of known mutations. However, it is not yet proven that oligogenicity is the primary mechanism in most patients with a familial history of disease and the aim of this study was to identify novel highly penetrant dominant Mendelian ALS genes, and so we believe that this is an appropriate strategy to adopt.

About 200 UK samples were previously exome captured in house by two fellow PhD students (Athina Soraya Gkazi and Jack W Miller) and sent for next generation sequencing at the Biomedical Research Centre (BRC) in Guy's and St Thomas' NHS Foundation Genomics Facility before I joined the laboratory. Exome capture and next generation sequencing for the remaining samples were outsourced by us or our collaborators. The design of capture probes remained consistent throughout the study.

2.1.2 Sporadic ALS (sALS) DNA samples

Some of the samples used in this study had no detectable family history and were, therefore, considered sporadic ALS cases (sALS). We had access to a cohort of 174 sporadic samples, which all had a definite ALS diagnosis that met the revised El Escorial criteria with full patient consent. These samples were Sanger sequenced in house in order to determine the frequency of mutations for each candidate ALS gene as a secondary cohort of cases, described in this thesis, which was discovered through exome sequencing of fALS cases. DNA was extracted from post mortem brain tissue supplied by the MRC London Degenerative Diseases Brain Bank.

2.2 Whole Exome Sequencing and data analysis

Whole Exome Sequencing (WES) allows the sequencing of all coding exons within the genome (exome). This process happens in two stages: stage one where specific probes isolate and purify known coding sequences from sonicated genomic DNA and stage two whereby whole exome is sequenced using Illumina sequencing by synthesis technology. This technique is a cost-effective way of identifying novel pathogenic variants. The exome, in fact, equals less than 2% of the whole human genome and this portion contains ~85% of the disease causing variants discovered to date in Mendelian diseases (Choi *et al.*, 2009).

2.2.1 Exome capture

The exome capture step was partly (~200 fALS UK samples) performed by my fellow PhD students Jack W Miller and Athina Soraya Gkazi, and partly outsourced by us and our collaborators. This was carried out making sure that the methodology and the design of capture probes was consistent throughout the study.

The exome capture procedure was carried out following the Illumina HiSeq protocol (Illumina TruSeq DNA Sample preparation guide) adapted to Nimblegen SeqCap EZ Exome library (Roche) version 3.0 (2012). This version comprises over 300,000 exons covering over 20,000 genes in the human genome (from RefSeq RefGene CDS (GRCh37), CCDS, miRBase v14 databases, 97% Vega, 97% Gencode, and 99% Ensembl databases) with a total size of the region covered by the probes of 64 Mb. An initial concentration of 5μg of DNA was processed. After each step the DNA was purified using SPRI Ampure XP beads (Beckman Coultier) and measured using Agilent Bioanalyser (Agilent 1000 DNA chip, Roche diagnostics) to check the quality and the amount of DNA purified.

Once the samples were captured, these were subjected to PCR in order to amplify the captured DNA. The samples were then washed using Qiagen Elute protocol (Qiagen, 28106), eluted in 50 μl of double distilled water ($_{dd}H_2O$) and ran on an Agilent chip in order to verify if the hybridisation with the probes was successful. If this was the case, samples were quantified using Qubit Fluorometer (Life Technologies), diluted to a concentration of 10pM and sent out for next generation sequencing.

2.2.2 Next Generation Sequencing

Samples were sent to the Biomedical Research Centre (Genomics Facility) in Guy's and St Thomas' NHS Foundation Trust where they performed next generation sequencing using Illumina platforms (HiSeq2000 and HiSeq2500 machines and in a minority of cases the GAII platform). Once the sequencing was completed we obtained the HiSeq

raw sequencing data, and these were converted into FASTQ formatted files, which were further analysed using the Illumina supplied program bcl2fastq.

2.2.3 Bioinformatic analysis of WES

The bioinformatics analysis was performed by my colleagues and bioinformatician Simon Topp and Michael Simpson. Novocraft NovoAlign was used to align the FASTQ files to the hg19 human reference and variants called with SAMtools v1.1 mpileup producing Variant Call Format (VCF) files, which were then normalised with bcftools v1.1 norm. Common ancestry between samples was taken from existing familial annotation where available and also deduced from IBD analysis in PLINKv1.07 (Purcell *et al.*, 2007). VCF files were filtered by the following criteria: DP≥10, QUAL>20, GQ≥50, and MQ≥50. Functional annotation, pathogenicity predictions, AdaBoost (ADA) & Random Forest (RF) splicing predictions (Jian, Boerwinkle and Liu, 2014) and matches to 1000 genomes were added with table_annovar.pl (Wang, Li and Hakonarson, 2010), whereas all other annotations including variant frequencies in ESP (http://evs.gs.washington.edu/EVS), ExAC (http://exac.broadinstitute.org) and UK10K (www.uk10k.org), were added via custom perl scripts. Variants were removed if they had a carrier frequency of greater than 1 in 20,000 in the Non-Finnish European subset of ExAC (MAF>0.0025%) or were predicted benign by at least 15 of the 20 pathogenicity prediction algorithms. Synonymous and intronic variants were assessed by NetGene2 and GeneSplicer and excluded if no changes in scores were observed compared to the reference allele at locations matching to known Refseq acceptor or donor splice sites. 5' and 3' UTR variants were excluded from consideration in this analysis.

2.3 Genetics

2.3.1 Primer design

Primers were designed using Primer3 version 4.0.0 (http://primer3.ut.ee/) using a DNA sequence template imported from Ensembl Genome Browser 91

(http://www.ensembl.org/index.html). They were designed so that they were as specific as possible to the template, with a GC content between 40% and 60% and, when possible, between 18-24 base pairs. All primers used in this study are listed in Appendix A and section 2.3.10. Primers were synthesized by Eurofins MWG (Germany) or Integrated DNA Technologies (IDT, Belgium). On arrival, primers were diluted and used at a working concentration of 10 μM. The optimal annealing temperature was determined by gradient PCR using high quality control PCR sample as template. Gradient PCR follows a PCR protocol similar to the one illustrated in section 2.3.2 with the difference that it uses a different annealing temperature on each one of the 12 columns of a 96 well plate with a range that goes from 50 to 68 °C.

2.3.2 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction is a technique developed by Kary Mullis and colleagues in 1980s (Mullis *et al.*, 1986). This technique uses the enzyme DNA polymerase to copy a desired fragment of DNA exponentially. The most commonly used enzyme is *Thermus aquaticus* (*Taq*) Polymerase, an enzyme extracted from a thermo-resistant bacterium. This enzyme generates fragments with A-overhangs and, in this study, it was widely used for cloning and Sanger sequencing. Other enzymes are also used as the Pyrococcus-like Phusion, which has a lower error rate due to its proof reading function and generates blunt end fragments. This was used for the amplification of larger, GC rich or more problematic fragments.

Different protocols were used for either Taq or Phusion polymerase as illustrated below (Table 2.2 and Table 2.3).

Component	Volume (μl) for one sample
dH20	13.4
10x buffer	2.5
$MgCl_2$ (50 mM)	1.0
dNTP (5mM)	1.0
Primer F (10 pmol/μl)	1.0
Primer R (10 pmol/μl)	1.0
Taq polymerase	0.1
Total Volume	**20**

Table 2.2 – Reagents for Taq polymerase PCR reaction. All products were purchased from Thermo Fisher

Component	Volume (μl) for one sample
dH20	13.4
HF 5x buffer	2.5
dNTP (5mM)	1.0
Primer F (10 pmol/μl)	1.0
Primer R (10 pmol/μl)	1.0
Phuison polymerase	0.1
10% enhancer	2.5
Total Volume	**20**

Table 2.3 - Reagents for Phusion polymerase reaction. All products were purchased from New England Biolabs

In the Phusion polymerase reaction a PCR enhancer (Thermo Fisher) was used at 10% final concentration in order to facilitate the expansion (see Table 2.3).

The reagents were mixed together and aliquoted in equal volume (20 μl) in a 96 well plate PCR together with DNA (concentration range 10-50 ng depending on availability and aim of the PCR).

The thermal cycling conditions for Taq and Phusion polymerase and are illustrated in Table 2.4 and Table 2.5.

Step	Temperature	Time	Cycles
First denaturation	95 °C	5 min	1
Denaturation Primer annealing	95 °C 50-68 °C 68 °C	30 sec 30 sec 1 min/kb	 35
Final extension	72 °C	7 min	1
Hold	4 °C	Until needed	

Table 2.4 - Thermal cycling conditions for the Taq PCR protocol.

Step	Temperature	Time	Cycles
First denaturation	98 °C	30 sec	1
Denaturation Primer annealing	98 °C 50-68 °C 72 °C	15 sec 30 sec 1 min/kb	 35
Final extension	72 °C	10 min	1
Hold	4 °C	Until needed	

Table 2.5 - Thermal cycling conditions for the Phusion PCR protocol

Thermal cycling was carried out on Tetrad-2 Peltier Thermal cycler (Bio-Rad) or MJ Research PTC-225 Gradient Thermal Cycler (Marshall Scientific).

2.3.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to check the quality, abundance and specificity of DNA available after PCR amplification.

Agarose (Helena Bioscience) was melted in 1x tris-acetate-EDTA (TAE) buffer in a concentration dependent on the size of the amplicon. 2% agarose gels were used for amplicon of 200-800 bp 1% agarose gels were used for amplicons > 800 bp. The molten agarose was left to cool and an aliquot of Ethidium Bromide (EtBr) was added to a final concentration of 100 μg/ml, EtBr intercalates between the DNA strands allowing the DNA to be visible under UV light, fluorescing at a wavelength of 302 nm. The gel was left to

cool in a mould with an appropriate number of combs inserted. DNA samples were mixed with 6X loading dye (Life technologies) and ran for a ranging time (20min-2hr) and voltage (120-180 V) depending on the size of the amplicon. For amplicons < 1000 bp a 100 bp DNA ladder was used (TriDye 100 bp DNA Ladde, NEB), for amplicons > 1000 bp a 1kb DNA ladder was used (NEB) in order to verify the fragment's size.

Agarose gels were imaged with 2UV Benchtop trans illuminator (UVP).

2.3.4 Gel purification

When needed, DNA was extracted from the agarose gel. The desired band was cut on a UV panel and stored in an Eppendorf tube. The DNA was extracted using QIAquick Gel Extraction Kit (Qiagen) following manufacturer's protocol. Concentration and quality of DNA was verified using Nanodrop 2000 spectrophotometer (Thermo scientific) as explained in section 2.3.7.

2.3.5 Sanger sequencing

Sanger sequencing is a DNA sequencing method that relies on the incorporation of fluorescently labelled modified oligonucleotides (with a dideoxynucleotide substitution) that cause a premature termination of a polymerase chain reaction.

2.3.5.1 Purification of PCR product

Successful Sanger sequencing requires a pre-cleanup stage in order to remove excess buffers, dNTPs, salts, primers and enzyme, and purify the PCR product that contains the fluorescent dideoxynucleotides (ddNTPs). The PCR product was, therefore, cleaned using Microclean (Microzone Ltd, UK). An equal volume of diluted DNA (1:2 or 1:3 in water depending on the abundance of PCR product) was added to Microclean (usually 6 μl) in a 96 well plate and spun for 40 minutes at 2,442g. The supernatant was then decanted spinning the plate upside down for 15 seconds at 449g, leaving a purified product ready for the Sanger sequencing reaction.

2.3.5.2 Sequencing reaction

BDT premix, composed by Big Dye Terminator V1.1 (Life Technologies) mixed with 5X sequencing buffer (Life Technologies), was added to the PCR products in order to prepare the samples for the sequencing reaction. Big Dye V1.1 contains a mix of the fluorescent ddNTPs and DNA polymerase as well as other reagents in a recipe optimised for the sequencing reaction. Either a forward primer or a reverse primer was added to this mix (volumes illustrated in Table 2.6). The primer was chosen considering the distance from the desired sequence (30 bp <distance< 200 bp) and the amount of T bases or A bases separating the primer from the desired sequence (not more than 7 of consequent As or Ts).

Reagent	**Volume (μl) 1X**
ddH_2O	6.8
BDT premix	3
Primer (10 pmol/μl)	0.2
Total	**10**

Table 2.6 - Volumes added to the sequencing mix.

The sequencing reaction is similar to a PCR reaction (protocol shown in Table 2.7) with the difference that, when a ddNTP (ddA, ddC, ddG, ddT) gets incorporated in the sequence, the extension is terminated. This results in the formation of amplicons of different lengths with ddNTPs at the 3' end.

Temperature (°C)	**Time**	**Cycles**
96	1 min	1
96	30 sec	28
50	15 sec	
60	4 min	
20	2 min	1
4	Until needed	-

Table 2.7 - Thermo cycling for sequencing reaction.

2.3.5.3 Purification of sequencing PCR products

Following the sequencing reaction, the PCR products were cleaned from all the excess reagents to avoid unspecific signal and damage to the machine. The samples were cleaned through two steps. In the first step samples were cleaned with ethanol (100%) complemented with 8% EDTA 125 mM, 32.5 μl of the mix were added to each well. The plate was centrifuged at 2,442g for 40 minutes. Successively, the supernatant was removed spinning the plate upside down at 449g for 15 seconds. In the second step a solution of 70% ethanol in double distilled water was added to each sample in a volume of 50 μl/well and spun at 543g for 20 minutes to precipitate the extension products. Supernatant was removed as described above and 10 μl/well of highly deionised Formamide (Hi-Di Formamide, Life Technologies) were added. The plate was then heated at 95 °C for 2 minutes to resuspend and denature the extension products.

2.3.5.4 Capillary electrophoresis

The plate was loaded on an ABI3130 (3130 Genetic Analyzer, Applied Biosystems (ABI) Ltd). The extension products, resuspended in formamide, were injected electrokinetically in capillaries filled with polymer (POP7, Life Technologies). A high voltage is applied so that the DNA fragments migrate towards the cathode depending on their size. This technique can distinguish between molecules that differ by one dNTP. These fragments move through a laser beam that will excite the fluorescent portion of the ddNTPs. Each ddNTP (ddA, ddC, ddG, ddT) will be conjugated to a different fluorophore. The machine reads this fluorescence translating it as chromatograms sequences represented by 4 colour peaks each characteristic of each base (A=green, C=blue, G=black, T=red).

2.3.5.5 Analysis of the sequencing data

Raw data was extracted from ABI3130 in the form of chromatogram .ab1 files and analysed using Sequencher 5.1 (GeneCodes Corporation, MI, USA). The .ab1 files were aligned against the reference sequence downloaded as FASTA format from Ensembl or

UCSC. Chromatograms were assembled into contigs and scrutinised by eye to identify any variant. Any variant found was investigated further in genome browsers (i.e. Ensembl and UCSC). No novel variant was identified in this study by Sanger sequencing.

2.3.5.6 Preparation of plasmid DNA for sequencing

As plasmid DNA is already pure and in high concentrations, it was diluted to a concentration of 10 ng/μl. 6μl were aliquoted in each well and 4 μl of sequencing mix was added to each sample. The sequencing mix was prepared as follows:

Reagent	Volume (μl) 1X
BDT Premix	3
Primer (10pmol/μl)	0.2
ddH_20	0.8
Total	**4**

Table 2.8 - Volume added for plasmid sequencing PCR.

Samples were then spun down, processed through sequencing PCR (as described in section 2.3.5.2) and, from this point, treated identically to PCR products.

2.3.6 Total RNA extraction from primary cell lines

Patient and control derived fibroblasts ($5x10^6$ cells), limphoblasts ($1X10^7$ cells) or HEK293T ($1X10^7$ cells) cells were harvested and washed once in PBS. The total RNA was extracted using RNeasy Mini Kit (Qiagen, cat no 74104) according to manufacturer's protocol with some variations. Cells were resuspended on ice using the appropriate amount of buffer RLT, they were then lysed using a 1 ml syringe attached to a 27 Gauge needle. The lysate was resuspended in 70% ethanol and transferred in a RNeasy Mini spin column placed in a 2 ml collection tube and spun at 8000 g for 15 seconds. The flow through was discarded and the column was washed with 350 μl of RW1 buffer prior treatment with RNAse free DNAse set (Qiagen) according to manufacturer instructions. This treatment uses DNAse I mixed with RDD buffer to eliminate any trace of DNA

contamination. The RNA was then washed in RPE buffer twice and eluted in 30-50 μl of RNAse-free water in an RNAse free tubes (Ambion).

2.3.7 Quantitation and purity assessment of nucleic acids

The concentration and quality of nucleic acid was assessed using Nanodrop 2000 spectrophotometer (Thermo Scientific). The purity of the nucleic acid was checked using the absorbance ratio 260/280 and 260/230. Nucleic acids absorb light at 260 nm, therefore, the ratio absorbance 260/280 was used to measure the level of protein (absorb light at 280 nm) contamination and it was considered pure if ~ 1.8 for DNA and ~ 2.0 for RNA. Absorption at 230 nm was used to detect contamination by organic compounds (i.e. ethanol contamination) and both DNA and RNA were considered pure when a ratio ≥ 2.0 was displayed. No samples were considered for any study if the quality values veered below the rations given above.

2.3.8 First strand cDNA synthesis

The extracted RNA was used as a template for the synthesis of complementary DNA (cDNA) through reverse transcription, using SuperScript® III Reverse Transcriptase (Life Technologies, cat no 18080044). Up to 1 μg of RNA was mixed with 1 μl of 50 μM oligo dT (deoxy-thymine nucleotides), 1 μl of annealing buffer and necessary amount of RNAse-free water to bring the solution up to 8 μl. Oligo dT is a mix of specific primers for the polyA tail of mRNA which allows transcription of the mRNA only, starting from the 3' of the transcript. The master mix was incubated at 65°C for 5 minutes and immediately placed on ice for at least 1 minute. Then 10 μl of 2x reaction mix and 2 μl Superscipt III/RNAseOUT enzyme mix was added to each sample. For each reaction at least one negative control with no enzyme added was included. The samples were then placed on the thermal cycler at 25 °C for 10 minutes followed by 50 minutes at 50°C terminating the reaction with 5 minutes at 85°C. Samples were stored at -20 °C.

2.3.9 Genomic DNA contamination and cDNA synthesis test

After cDNA synthesis ~ 25ng of newly synthesised cDNA were amplified by PCR using *GAPDH* primers (Table 2.9) in order to verify if the cDNA synthesis had been successfully completed. The samples were run alongside at least a negative control (RNA reverse transcribed with no enzyme) and a positive control (control DNA) to check for the efficiency of the *GAPDH* PCR. Samples were then run on a 2% agarose gel (see section 2.3.3) to check if the newly synthesised samples had been amplified and if the negative control showed any DNA contamination.

2.3.10 Quantitative real-time PCR

2.3.10.1 Primer design

Primers were designed as described in section 2.3.1 with the difference that the quantitative PCR (qPCR) primers design was optimised for a fragment of 100 bp. When possible, they were designed to map on exon extremities overlapping a large amount of intronic DNA, so that they would not amplify genomic DNA if present. *GAPDH* primers did not span exon boundaries as they were used for genomic DNA contamination test. All the primers used for qPCR in this study are illustrated in Table 2.9.

Gene	Forward primer	Reverse primer
GAPDH	ATCATCAGCAATGCCTCCTGC	ATGGCATGGACTGTGGTCATG
RPL13A	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTC
SDHA	AGGAATCAATGCTGCTCTGG	CTGCTCCGTCATGTAGTGGA
OPTN	GTCCTTGATGGAGATGCAGAG	GGGCAGGAATGAATCGGAATA

Table 2.9 - Sequence of the qPCR primers used in this study.

2.3.10.2 qPCR protocol

All the samples tested for the same gene were included on the sample plate following the sample maximisation approach (Hellemans *et al.*, 2007). cDNA samples were diluted to a concentration of 5 ng/μl and 4 μl of each sample (total of 20 ng) were aliquoted in a

white welled 96 well plate (Thermo Scientific) in technical triplicates. The samples were mixed with primers at a concentration of 0.2 μM, 1X FastStart Universal SYBR Green Master (Rox) (ROCHE) diluted to a volume of 20 μl with RNAse free water. The protocol used for qPCR is illustrated below (Table 2.10).

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	15 minutes	1
Denaturation	95	30 seconds	45
Annealing	60		
Extension	72		

Table 2.10 - Thermo cycling for qPCR reaction.

For each cycle fluorescence was recorded. A melting curve was developed recording a plate read every 1 ºC increase from a temperature range between 60-95 ºC. All the samples were run on a Chromo4™ Real-Time PCR detector (Bio-Rad). Furthermore, all experiments were carried out in technical and biological triplicates to correct for technical and biological variability. The same batch of FastStart Universal SYBR Green Master (Rox) was used within the same experiment to reduce variability. For every plate ran, two negative controls we added where RNAse free water replaced DNA. For every sample a total of three housekeeping genes (*GAPDH*, *RPL13A*, *SDHA*) (Table 2.9) were run and used as reference genes in the data analysis process.

2.3.10.3 qPCR data analysis

Opticon Monitor™ (Bio-Rad) was used for a first analysis of the data. Using this software, a threshold was selected (placing it in a region where all the curves were parallel) and melting curve assessed. Data files were then exported to Excel 2017 (Microsoft), more specifically cycle threshold (CT) and efficiency values were exported. Technical replicates were averaged to values representing the biological replicates, used for statistical analysis. The analysis was carried out applying the Pfaffl mathematical model for relative transcript quantification (Pfaffl, 2001), which formula is illustrated below:

$$Ratio = (E_{target})^{\Delta CT_{target}(control-sample)} \div (E_{ref})^{\Delta CT_{ref}(control-sample)}$$

Equation 1 - Pfaffl equation. ΔCT_{target} = target gene cycle threshold, ΔCT_{ref} = mean of reference gene CTs, and E = PCR efficiency.

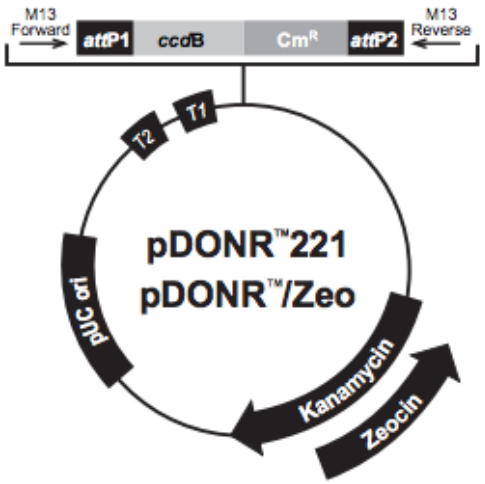
The aim of this analysis is to enable a relative quantification of the target gene compared to an endogenous standard, represented by the reference genes.

2.4 Plasmids and Cloning

2.4.1 Gateway Cloning

2.4.1.1 pDONR reaction

A *MATR3* construct was ordered from Creative Biogene Biotechnology untagged and inserted in the donor vector pDONR221 (Figure 2.1). Primers were designed following the procedure illustrated in section 2.3.1 in order to insert an HA tag at the N-terminus of *MATR3*. These primers included the following sequences in-frame, firstly a comprehensive of HA tag sequence (on the Forward (5') primer), followed by the Kozak translation initiation sequence and restriction enzyme sites (attB1 on the Forward (5') and attB2 on the Reverse (3') primers).



Comments for:	pDONR™221 4761 nucleotides	pDONR™/Zeo 4291 nucleotides
*rrn*B T2 transcription termination sequence (c):	268-295	268-295
rrnB T1 transcription termination sequence (c):	427-470	427-470
M13 Forward (-20) priming site:	537-552	537-552
*att*P1:	570-801	570-801
*ccd*B gene (c):	1197-1502	1197-1502
Chloramphenicol resistance gene (c):	1825-2505	1847-2506
*att*P2 (c):	2753-2984	2754-2985
M13 Reverse priming site:	3026-3042	3027-3043
Kanamycin resistance gene:	3155-3964	---
EM7 promoter (c):	---	3486-3552
Zeocin resistance gene (c):	---	3111-3485
pUC origin:	4085-4758	3615-4288
(c) = complementary strand		

Figure 2.1 - pDONR221 and pDONR/Zeo vector map (Life Technologies)

These primers were used to amplify the construct through PCR as described in section 2.3.2. After PCR the amplicon would contain the N-terminal HA tag, Kozak sequence and attB1 and attB2 restriction sites that can be ligated into pDONR221 vector. This was done using the Gateway Cloning Protocol (Life Technologies). This includes a BP reaction, mixing the enzyme BP clonase with the pDONR221 (~75ng) and the amplicon (~75 ng) at 25 °C for 1 hour, which would cut the pDONR221 on the attP restriction sites. These are complementary to the attB restriction sites and recombine with those so that the amplicon can be inserted in the pDONR221. Once the reaction had taken place, the samples were treated with Proteinase K for 10 minutes at 37 °C to terminate the reaction and transformed into bacteria as described in section 2.4.1.2.

2.4.1.2 Bacteria transformation and plasmid extraction

Once the reaction was terminated, 1 µl of the product was added to 20 µl of Max Efficiency® DH5α™ competent cells (Invitrogen) and they were incubated for 10-30 minutes on ice. The samples were then placed at 42°C for 30 seconds and back on ice for 2 minutes, in order to heat shock the cells and aid the transformation. Super Optimal broth with Catabolite repression (S.O.C.) Medium (Invitrogen) was added to the cells and the samples were shaken at 37°C for one hour. The transformed bacteria were plated on LB Agar (Sigma-Aldrich) plates with 0.05 mg/ml Kanamycin (Bioline) or 0.1 mg/ml Ampicillin (Bioline). Bacteria were left growing overnight at 37°C. Colonies were picked and added to LB broth (Sigma-Aldirch) complemented with 0.05 mg/ml Kanamycin (Bioline) or 0.1 mg/ml Ampicillin (Bioline). The samples were left shaking at 37°C overnight and the morning after the bacteria were mini-prepped with plasmid purification kit (Macherey-Nagel) following manufacturer's instructions. DNA concentration was determined using Nanodrop 2000 (Thermo Scientific).

2.4.1.3 pDEST reaction

The second step of the gateway cloning is inserting the product in an expression vector (pDEST, Invitrogen). After extraction, 75 ng of the entry clone (pDONR comprising of the PCR product), was combined with pT-Rex-DEST30 (Life Technologies, 75 ng/µl) illustrated in Figure 2.2, TE buffer and LR Clonase™ II (Life Technolgies) and incubated at 25°C overnight. The enzyme LR Clonase catalyses the combination reaction that flips the construct to the destination vector using the attL restriction sites. The following day proteinase K was added, transformation and plasmid extraction was performed as explained in section 2.4.1.2. The construct was then ready to be expressed.

Map

The map below shows the elements of pT-REx-DEST30. DNA from the entry clone replaces the region between bases 706 and 2389. **The complete sequence of pT-REx-DEST30 is available from our Web site (www.invitrogen.com) or by contacting Technical Service (page 21).**

Comments for pT-REx-DEST30
7544 nucleotides

CMV promoter: bases 1-588
TATA box: bases 502-508
Tetracycline operator (2X $TetO_2$) sequence: bases 518-557
*att*R1 recombination site: bases 699-823
Chloramphenicol resistance gene: bases 932-1591
*ccd*B gene: bases 1933-2238
*att*R2 recombination site: bases 2279-2403
T7 promoter: bases 2464-2483 (complementary strand)
SV40 polyadenylation region: bases 2915-3045
f1 origin: bases 3175-3603
SV40 early promoter and origin: bases 3790-4098
Neomycin resistance ORF: bases 4157-4951
Polyadenylation region: bases 5015-5063
Ampicillin (*bla*) resistance ORF: bases 5474-6334
pUC origin: bases 6479-7152

Figure 2.2 - pT-Rex_DEST30 vector map (Life Technologies)

In the interest of time, HA-tagged *TBK1* wild type (NM_013254.2, Figure 2.4) and FLAG-tagged *OPTN* (BC013876, Figure 2.3) were custom made and purchased from Sino Biological (Cat no HG11023-NY and HG14478-NF, respectively).

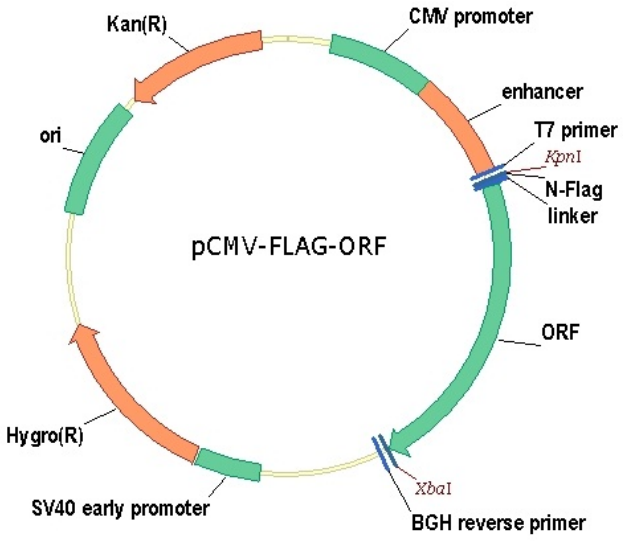


Figure 2.3 - pCMV-FLAG-ORF backbone map

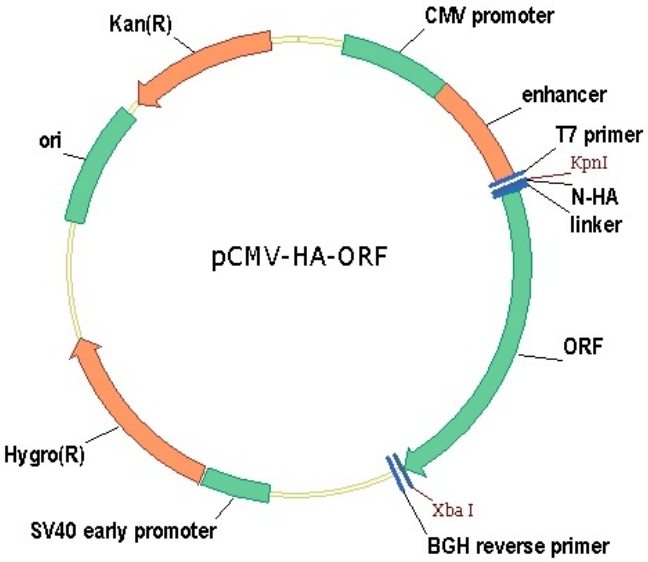


Figure 2.4 - pCMV-HA-ORF backbone map

2.4.2 Mutagenesis

All the variants analysed in the current study were modelled using Q5® Site-Directed Mutagenesis (New England Biolabs). This kit is designed for rapid incorporation of insertion, deletions and single nucleotide changes on double stranded plasmid DNA.

2.4.2.1 Mutagenesis primer design

In order for modifications to be incorporated in the plasmids, primers were designed using the QuikChange Primer Design platform provided by Agilent (http://www.genomics.agilent.com/primerDesignProgram.jsp). Primers were then ordered as described in section 2.3.1 and they are listed in Table 6.2.

2.4.2.2 Mutagenesis protocol

As specified above all the mutagenesis reactions were carried out using Q5® Site-Directed Mutagenesis (New England Biolabs) according to manufacturer's instructions. Briefly, this kit includes a Q5 Hot Start High fidelity DNA polymerase, which exponentially amplifies the template using custom designed primers (see section 2.4.2.1). The

amplicons are then incubated with a mix of kinase, ligase and DpnI that facilitates the circularisation of the PCR products. The resulting plasmids are then transformed into bacteria, plated on agar plates and extracted (if the reaction is successful) as illustrated in section 2.4.1.2. This process is illustrated in Figure 2.5.

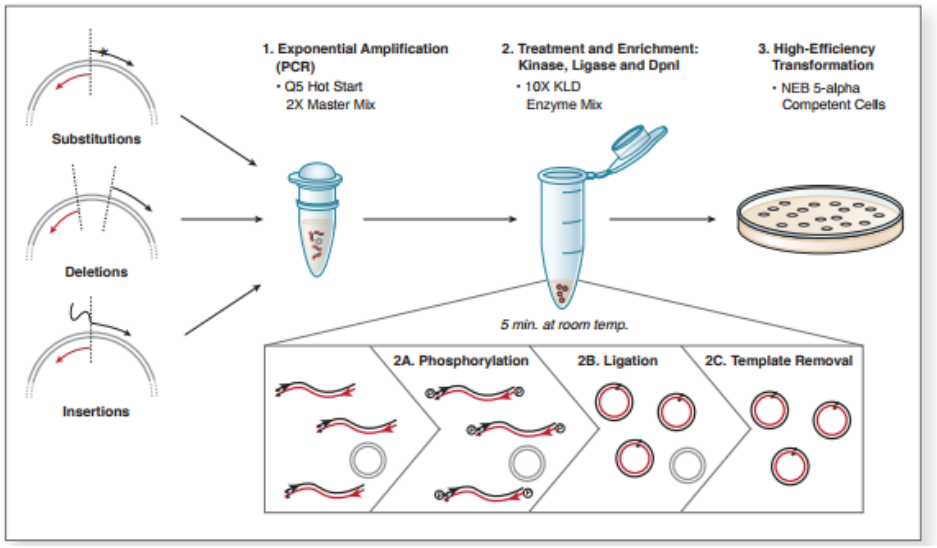


Figure 2.5 - Q5 site directed mutagenesis kit overview (NEB).

Once extracted, the plasmids were quantified (section 2.3.7) and sequenced verified in house by Sanger Sequencing as illustrated in section 2.3.5. If the mutagenesis reaction was successfully confirmed by sequencing, the plasmids were ready to be transfected in mammalian cells.

2.5 Cell culture

2.5.1 Human Embryonic Kidney cells 293T (HEK293T)

HEK293 cell line was generated for the first time in 1977 by Graham and Smiley (Graham, 1977). It is derived from human embryonic kidney cells and it is widely used for biochemical assays. In this study, we use the HEK293T cell line (DuBridge *et al.*, 1987), which expresses the SV40T antigen that allows transfected plasmid that carry SV40T origin of replication to be amplified, making this cell line particularly valuable for overexpression studies.

2.5.1.1 Maintenance of HEK293T

HEK293T cells were grown in adhesion in Nunc 175cm Flask (Fisher) in Dulbecco's Modified Eagle Medium (DMEM) complemented with 4.5g/L D-Glucose (Life Technologie), 10% FBS (Fetal Bovine Serum, Life Technolgies), 5% PenStrep (penicillin 100 U/ml and streptomicin 100 U/ml, Life Technologies) and 5% L-Glutamine (Life Technologies). The flasks were kept in a water-jacketed 5% CO_2 incubator.

Once the cells reached confluence they were passaged by removing the old media, washing the adherent cells with Dulbecco's phosphate-buffered saline (DPBS, Life Technologies) and incubating the cells for 3 minutes at room temperature with 1 ml of Trypsin/EDTA solution (TE, Life Technologies). TE is a mixture of proteases derived from bovine pancreas widely used to dissociate cells. Once the cells were detached and floating in the TE, 9 ml of complemented DMEM were added to the flask. The FBS in the media inhibits the action of the proteases, this avoids any damage to the cells, which would happen if the cells were kept in TE for a long time. The mixture of media and cells was then pipetted up and down a few times to make sure that the cells were correctly dissociated and only 1/10 of the mixture was plated in the flask and ~10 ml of media were added to it. The rest of the cells were either thrown away or used for plating.

All the reagents used in this process were heated at 37ºC for at least 30 min before being used in order to avoid any heat shock to the cells.

2.5.1.2 Plating of HEK293T

Cells were plated using a method based on the plate surface area. According to this method the surface area of the desired well in cm^2 (i.e. 6 well/plate, 24 well/plate, 96 well/plate) was multiplied by the desired confluency (i.e. 25% for transfection experiments). The obtained number was then multiplied by the amount of wells to be plated (plus one extra to make sure to have enough cells) resulting in the total surface area to be plated. The obtained number was then divided by the surface area of the flask (usually a T75), this number would give the amount (in ml) of cells needed in a fully

confluent flask. The number obtained was multiplied by 10 which was the amount of liquid present in the flask. This number was then detracted from the final amount of media needed in total. All the numbers used in these calculations were taken from the Life Technologies website (Table 2.11)

Cluster Plates	**Surface Area (mm^2)**	**Growth Medium (ml)**
6 well	962	2-5
12 well	401	1-2
24 well	200	0.5-1
Flasks		
T25	2500	3-5
T75	7500	8-15
T160	16000	15-30

Table 2.11 - Surface area of plates and flasks used in this study (Life Technologies)

Below is an example of how to calculate the amount of cells needed (in ml) for a full 6 well plate at 25% confluency starting from a confluent t75 flask:

Surface area of one well of a 6 well plate = 9.62 cm^2

0.25 * 9.62 cm^2 = 2.4 cm^2

7 (wells) * 2.4 cm^2 = 16.8 cm^2

16.8 cm^2 / 75 cm^2 = 0.224 cells needed for a confluent T75 flask

0.224 * 10 ml = 2.24 ml of cells

2ml (volume of media needed per well) * 7 (number of wells wanted) = 14 ml

14 ml – 2.24 ml = 11.76 ml of media needed

Usually for immunocytochemistry (ICC) a 24 well plate would be used whereas for western blotting and other biochemical assays a 6 well plate would be used.

2.5.2 SH-SY5Y cell line

Derived from the bone marrow of a 4 year old neuroblastoma patient SH-SY5Y cell line was described for the first time in 1978 (Biedler *et al.*, 1978). This cell line has neuronal characteristics such as neuron outgrowth and dopamine activity. These characteristics make the cell line a better model than HEK293T for the study of disorders of neurological nature. However, these cells are more difficult to grow and transfect and, therefore, less useful for biochemical assays.

2.5.2.1 Mantainance of SH-SY5Y

SH-SY5Y cells were grown in adhesion on T75 flasks (Nunc, Life Technologies) in DMEM-F12 medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, Life Technologies) complemented with 4.5g/L D-Glucose (Life Technologie), 10% FBS (Fetal Bovine Serum, Life Technolgies), 5% PenStrep (penicillin 100 U/ml and streptomicin 100 U/ml, Life Technologies) and 5% L-Glutamine (Life Technologies). The flasks were kept in a water-jacketed 5% CO_2 incubator.

Cells were passaged as described in section 2.5.1.1 with the exception that instead of DMEM, complemented DMEM/F-12 was used.

2.5.2.2 Plating of SH-SY5Y

SH-SY5Y were plated using the plate surface area method explained in section 2.5.1.2 with the exception that SH-SHSY grow much slower and were initially plated at a higher confluency (~30%).

2.5.3 Cell transfection

HEK293T and SH-SY5Ycells were mainly used for overexpression studies. 24 hours after plating these cells were transfected with the appropriate plasmid using Lipofectamine 2000 (Life Technologies) and OPTIMEM (Life technologies) using the following protocol:

For a 6 well plate:

50 µl of OPTIMEM per sample were mixed with 2 µl of Lipofectamine per sample (i.e. for 6 samples 300 µl of OPTMIMEM were mixed with 12 µl of Lipofectamine), spun down and left to rest for 5 minutes.

In the meantime, for each sample, 1 µg of DNA was mixed with 50 µl of OPTIMEM.

50 µl of the OPTMIMEM and Lipofecatmine mix was pipetted in each sample, vortexed, spun down and left to incubate at room temperature for 20-50 minutes.

100 µl for each sample were added to each well. Cells were harvested after 48 hours from transfection.

For a 24 well plate the process was very similar with the only difference being in the amount of reagents used: the total volume of OPTIMEM used was 25 µl, Lipofectamine was used at 0.5 µl/sample and the total amount of DNA used per sample was 250 ng.

Cells were usually transfected alongside an empty GFP clone used as a positive control for the transfection reaction.

2.5.4 Fibroblast primary lines

Through a collaboration with Doctor Marc Gotkine (Hadassah University Hospital, Israel) we obtained some Fibroblast primary lines from four members of a consanguineous Palestinian family affected by fALS and carrying a truncation mutation in OPTN (p.S174X). Skin biopsies were obtained in Shaare Zedek Medical Centre and fibroblasts were derived following a well-established protocol. Briefly, skin biopsies were minced and placed in a 35X10mm cell culture dish in 1 ml medium of BioAmph 2 (Biological Industries) in a 5% CO_2 incubator at 37ºC. The tissue would then attach to the dish and culture fresh media would be added every other day. After a period of 1-3 weeks fibroblast would start to attach to the bottom of the well. When 70% of confluency was reached, the cell would be trypsinised, harvested and frozen or re plated. Four lines were

generated from the family (three carrying a heterozygous truncated mutation on OPTN and one carrying a homozygous mutation). Additionally, four fibroblast control lines were kindly donated to us from Professor Fiona Watt's laboratory. These cells were commercially available Normal Human Dermal Fibroblasts (NHDF) derived from adult female provided by PromoCell (lot number 4032503.1, 4081903.2, 3102301.3, 4012203.1).

2.5.4.1 Maintenance of primary fibroblast lines

SH-SY5Y cells were grown in adhesion on T75 flasks (Nunc, Life Technologies) in DMEM complemented with 4.5g/L D-Glucose (Life Technologie), 20% FBS (Fetal Bovine Serum, Life Technolgies), 5% PenStrep (penicillin 100 U/ml and streptomicin 100 U/ml, Life Technologies) and 5% L-Glutamine (Life Technologies). The flasks were kept in a water-jacketed 5% CO_2 incubator.

Once the cells reached confluence they were passaged removing the media, washing the adherent cells with DPBS and adding 3 ml of TE (Life Technologies). The cells were put back in the incubator for ~5 minutes, the flasks were then gently tapped and, with the use of a bright field microscope, cells were examined to make sure that they detached from the bottom of the flasks. 4 ml of media was then added to each flask and the cells were resuspended in a mix of media and TE. The liquid was then removed from the flask and put in 15 ml falcons (Alpha). The falcons were then spun for 4 minutes at 1000 g, supernatant was removed, cells were resuspended in media and the appropriate amount of cells was replated in the flask. The rest of the cells were either plated (as described in section 2.5.4.2), processed for freezing (as described in section 2.5.6) or processed for functional studies (as described in section 2.6). Number and date of passage was noted on the flask.

2.5.4.2 Plating of primary fibroblast lines

Once the cells were pelleted and the supernatant removed, they were resuspended in 1-2 ml of medium. 19 µl of resuspended cells were mixed with 1 µl of Solution 13

(Chemotech, 30 µg/ml Acridine orange mixed with 100 µg/ml DAPI in water).10 µl per sample were pipetted in each chamber of a NC-Slide A8 (Chemotech) and loaded on NucleoCounter® NC-3000™, which is an image cytometer that uses fluorescence imaging to characterise cell properties. Cells were counted using the Viability and Cell Count Assay. This program uses the acridine orange staining to identify each cell and it uses the DAPI staining to detect the non-viable cells, as DAPI can not penetrate the cell membrane if the cell is healthy. This program reveals the amount of cells present in 1 ml. Fibroblasts were then plated in the correct amount. Usually ~10,000 cells/well were plated for 96 well plates, ~3,000 cells/well were plated for 24 well/plate (usually for immunocytochemistry) and ~100,000 cells/well were plated for 6 well/plate. The appropriate amount of media was then added to a volume of ~100 µl for a 96 well plate, 500 µl for a 24 well plate and 2 ml for a 6 well plate.

2.5.5 B-lymphoblastoid cell lines

B-lymphoblastoid cell lines were derived from fALS patients and healthy controls by the European Collection of Authenticated Cell Cultures (ECACC). Human B cells, extracted from patients' and healthy volunteers' blood, were infected with Epstein-Barr virus (EBV). EBV establishes a latent infection resulting in an immortalised cell line. The cell lines derived from patients have the advantage of having an endogenous expression of the variant of which the patient is carrier.

2.5.5.1 Maintenance of B-lymphoblastoid cell lines

B-lymphoblastoid cell lines were grown in RPMI media (Gibco, Life Technologies) complemented with 10% FBS (Fetal Bovine Serum, Life Technolgies), 5% PenStrep (penicillin 100 U/ml and streptomicin 100 U/ml, Life Technologies) and 5% L-Glutamine (Life Technologies). These cells grow in suspension and were, therefore, kept in upright T25 flasks (Nunc, Life Technologies) in a water-jacketed 5% CO_2 incubator.

These cells were passaged weekly by aspirating half of the flask content and replacing it with fresh media. The removed flask content was either thrown away or pelleted and

processed for RNA extraction (as described in section 2.3.6) or western blotting (as described in section 2.6.2).

2.5.6 Freezing and thawing cells

For freezing procedures all cells were harvested (trypsinised if they were growing in adherence), spun down at 1000g for 4 minutes and the supernatant removed. Cells were, then, resuspended in the appropriate amount of FBS complemented with 10% DMSO and 500 µl was transferred in each appropriately labelled cryotube (VWR). DMSO is a cryoprotective reagent that reduces ice crystal formation when the media around the cells freezes. Cryotube were then transferre to a Mr. Frosty™ Freezing Container (Thermo Fisher Scientific) and placed at -80 °C.

When thawed, cells were resuspended in the appropriate and preheated medium and transferred in falcon tubes, which were spun at 1000g for 4 minutes to eliminate any trace of DMSO, toxic for the cells. Supernatant was aspirated and the cells were resuspended in ~2 ml of appropriate media and, if grown in adherence, plated in a T25 flask until confluence.

2.6 Functional and biochemical assays

In this section I will describe all the functional assays used in this study to characterise the role of the genes and the variants studied.

2.6.1 Immunocytochemistry (ICC)

Immunocytochemistry (ICC) is a widely used technique that allows the visualisation, when observed through a fluorescence or confocal microscope, of cellular localisation and distribution of specific proteins when labelled with fluorescent antibodies.

For immunofluorescence, cells were plated in 24 well-plates on thirteen-mm-diameter, 1.5-mm-thick coverslips (VWR), these were treated with poly-D-lysine (Sigma-Aldrich), a positively charged polymer that strengthens the interaction between the cell membrane

and the culture surface, for an hour and then washed once with DPBS before plating. Depending on whether the cells had to be transfected (in the cases of HEK293T or SH-SY5Y) or not (in the case of primary fibroblasts) cells were fixed 48 hours or 24 hours after plating with 4% paraformaldehyde (PFA) at room temperature for fifteen minutes. They were then washed 3 times with DPBS, permeabilised with 0.2% Triton-X-100 (Sigma) for 30 minutes and blocked with 5% Goat or Donkey Serum (Sigma), depending on the choice of secondary antibody, for one hour at room temperature. Samples were incubated with primary antibody (Table 2.12) in 1% Goat Serum overnight at 4 °C. They were washed twice with DPBS for 10 minutes each time and incubated with fluorescent tagged secondary antibodies (Alexa Fluor 488 Goat IgG Antibody, Alexa Fluor 568 Goat IgG Antibody, Donkey Anti-Sheep IgG H&L (Alexa Fluor® 568),1/500 Life Technologies. Alexa Fluor® 647 AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L), Jackson Immuno Reseach 1/500) for fluorescence detection in 1% donkey or goat serum (the choice of serum has to stay consistent throughout the protocol) for 2 hours at room temperature in the dark. As a negative control samples were incubated with primary antibody only or secondary antibody only. Following two 10 minute washes with DPBS cells were incubated with DAPI (4', 6-diamidino-2-phenylindole) to detect the nuclei. Coverslips were washed twice in DPBS and mounted on microscope slides (Thermo Scientific) using FluorSave™ Reagent (Calbiochem). Cells were imaged using Leica confocal microscope (Leica).

Antibody	Cat n°	Antigen	Company	Host	ICC	WB
COXIV	4850	Residues surrounding Lys29	Cell Signalling	Rabbit	1:250	1:2000
DDK (FLAG)	TA5001	Whole protein	Origene	Mouse	-	1:3000
GAPDH	G8795	Whole protein	Sigma-Aldrich	Mouse	-	1:6000
HA-Tag	2367	Whole protein	Cell Signalling	Mouse	1:500	1:1000
HA-Tag	3724	Whole protein	Cell Signalling	Rabbit	1:500	1:1000
IRF3	A022993	Whole protein	Bioassay Technology Laboratory	Rabbit	1:100	1:200
NAK/TBK1	ab40676	N-t	Abcam	Rabbit	1:250	1:500
Optineurin	ab23666	aa 575-591	Abcam	Rabbit	-	1:200
Optineurin (C-term)	100000	aa 559-575	Cayman	Rabbit	-	1:500
Optineurin (INT)	100002	aa 115-130	Cayman	Rabbit	-	1:200 ECL
Phospho-IRF-3 (Ser396)	ab76493	Ser396	Abcam	Rabbit	1:200	1:200
Phospho-TBK1 (S172)	5483	S172	Cell Signalling	Rabbit	1:50	1:500
SQSTM1/p62	ab91526	14aa at C-t	Abcam	Rabbit	1:500	1:500
TDP-43	10782-2-AP	Non specified	Proteintech	Rabbit	1:500	1:1000
Tom-20	612278	aa 47-145	BD Transduction Laboratories	Mouse	1:250	1:1000
Ubiquitin, Lys63-specific	05-1308	Lys-63 ubiquitination site	Millipore	Rabbit	1:2000	1:2000

Table 2.12 - Table showing all the antibodies used in this study, their characteristics and their work concentration.

2.6.1.1 MitoTracker

MitoTracker (Thermo Fished Scientific, M7512) is a cell permanent probe which contains a mildly thiol-reactive chloromethyl moiety for mitochondria labelling. This technique was used on live fibroblasts.

Live cells were plated in 24 well plates as described in section 2.5.4.2 and 2.6.1 before fixation fresh media was added to the cells containing MitoTracker at a concentraton of 100nM. The cells were then put back in the incubator for 20 minutes. After the incubation

time cells were fixed, washed, incubated with DAPI and mounted on microscope slides as described in section 2.6.1.

2.6.1.1.1 MitoTracker analysis

Cells were imaged at Leica DM5000B microscope using a 63x HCX PL Fluotar phase objective (Milton Keynes, UK). Images were taken randomly across the coverslips being careful not to image the same section twice, 15 images were taken per sample.

The analysis was carried out using Fiji (ImageJ) (Schindelin *et al.*, 2012) more specifically, an ImageJ Mitochondrial Morphology macro developed by Ruben K. Dagda at the University of Pittsburgh (Dagda *et al.*, 2009). This analysis allows the identification of different morphologic characteristics of mitochondria such as area, solidity, circumference and average size (see Figure 2.6). In order to obtain an accurate identification of singular mitochondria a threshold has to be manually set, for this reason the analysis was carried out blinded. The data obtained from this analysis was further analysed using GraphPad as described in section 2.7.

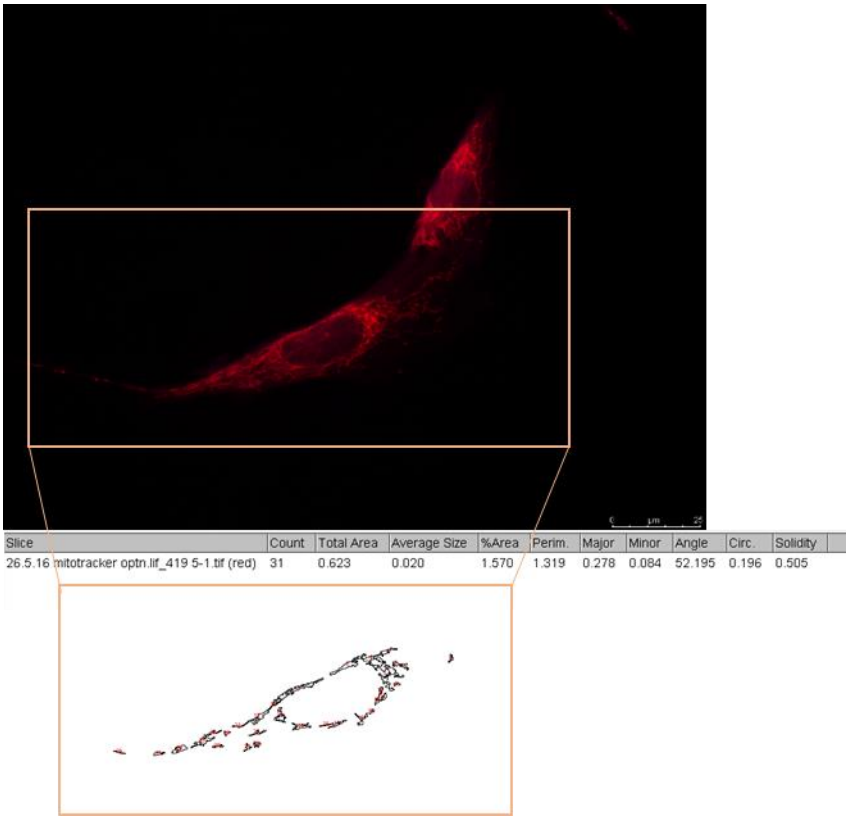


Figure 2.6 – Mitochondria morphology analysis. Representation of the mitochondria morphology analyses conducted on fibroblasts using the ImageJ macro developed by Ruben K. Dagda.

2.6.2 Western Blotting

Western blotting (or immunoblotting) is a widely used technique that allows identification and quantification of overexpressed or endogenous proteins through the use of an electrophoresis system and immuno-labelling.

2.6.2.1 Cell harvesting and protein quantification assay

Once the cells had been plated, and transfected when necessary, they were harvested using the appropriate buffer, depending on the required assay. For regular western blotting, cells were washed with PBS complemented with phosphatase inhibitors (PhoSTOP, Roche) and proteinase inhibitors (COMPLETE, Roche) and lysed in 1x lysis buffer (62.5 mM TRIS-HCL pH 6.8, 2% SDS, 1% glycerol). All the samples were sonicated for 10 seconds each. The amount of protein was quantified using the DC Protein Assay Kit II (BioRad) according to manufacturer's protocol. In order to have an

accurate measure of the protein concentration a standard curve was made using a ranging concentration of BSA (Bovine Serum Albumin, Fisher Scientific) from 0 to 2 µg/µl (total of 9 concentrations, in duplicate, 5 µl per well of a clear 96 well plate (Fisher)). 2.5 µl of lysate per sample was mixed with 2.5 µl of 1x lysis buffer in a 96 well plate and every sample was analysed in technical duplicate. Then 25 µl of a mix of Reagent A and Reagent S (50:1) and 200 µl of Reagent B were sequentially added to each sample, the plate was then left in the dark for 15 minutes. The absorbance was measured at a wave length of 450 nm using FLUOstar Omega plate reader (BMG Labtech). Protein concentrations were calculated on the base of the mean absorbance of sample duplicates in relation to the BSA standard curve.

2.6.2.2 Gel electrophoresis

Once the samples were quantified, 5-20 µg of protein was mixed with an appropriate amount of loading buffer (lysis buffer complemented with bromophenol blue for sample visualisation) to a volume of 15 µl. These samples were then boiled at 100 ºC for 10 minutes and loaded, alongside 1.5µl of Precision Plus Protein Dual Color Standard (BioRad) used to determine protein size, on a NuPAGE Novex 10% Bis-Tris Midi gels (26 wells). These were run in 1x Morpholinepropanesulfonic acid (MOPS, Life Technologies) buffer at a ranging voltage of 100-180 V until needed, depending on the level of size separation wanted. Furthermore, ice was added in a separate compartment to cool down the gel as the temperature increases during the electrophoresis process.

2.6.2.3 Protein transfer

Once the proteins in the lysate were separated via gel electrophoresis, they were then transferred on a nitrocellulose membrane in order to be detected by immunoblot. This was done using iBlot (successively upgraded to iBlot 2, ThermoFisher Scientific) dry blotting system according to manufacturer's instructions. Briefly, NuPAGE™ Transfer Buffer (20X) (ThermoFisher Scientific) was diluted in 50 ml of ultra-pure water and complemented with 10% methanol. The NuPAGE Novex 10% Bis-Tris Midi gel was

removed from the plastic container and washed in transfer buffer for 5 minutes, this was then positioned on the transfer stack and the iBlot was operated for 7 minutes. In this system, the proteins were transferred to the nitrocellulose membrane using a shortened gap between electrodes and high currents for 7 minutes, the proteins bind to the membrane thanks to hydrophobic and electrostatic interactions.

Following the transfer, the membrane was incubated with 1x Pounceau S (Sigma), which unspecifically weakly binds to any protein, for 1 minute to verify that the transfer had worked correctly. The membrane was then washed in TBS-T (50 mM Trizma base (Sigma), 150mM NaCl (Sigma) at a pH of 7.6 complemented with 0.05% Tween20 (Sigma)) until clear from Pounceau staining.

2.6.2.4 Immunoblotting

In order to avoid any unspecific binding of primary or secondary antibody to the membrane, this was blocked with either 5% fat free milk (Sigma) or, rarely, 5% BSA (Fisher Scientific) in TBS-T for 1 hour.

The membrane was then incubated overnight at 4ºC with primary antibody in a 1% fat free milk or BSA solution. The following day the membrane was washed in TBS-T three times and incubated with the relevant IRDye secondary antibodies (1:5000, Li-Cor) diluted in TBS-T complemented with 1% fat free milk or BSA for 1 hour at room temperature in the dark. The membrane was then washed three times and either scanned with the Odyssey Infrared Imaging System (Li-Cor Biosciences) or developed using ECL system (as described in section 2.6.2.4.1) to detect the fluorescent signal.

2.6.2.4.1 Enhanced chemiluminescence (ECL)

When the signal was predicted to be weak (mainly when detecting endogenous, lowly expressed proteins) enhanced chemiluminescence (ECL) method was used to detect fluorescent signal. In this technique horseradish peroxidase (HRP) secondary antibodies for mouse (Millipore, 12-349) or rabbit (Millipore, 12-348) were used at a concentration of

1:2000. Following washes, the membrane was treated with Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500) following manufacturer's instructions. The mix of reagents was distributed on the membrane and excess reagent removed. Following incubation in the dark for 2 minutes, the membrane was inserted in an autoradiography cassette and carried to a dark room. Here, a radiography film (VWR International, 28-9068-36) was left exposing for the appropriate amount of time (depending on signal intensity) and developed using Medical Film Processor SRX-101A (Konica Minolta).

2.6.2.5 Western blot quantitative analysis

Western blots were quantified using Fiji (Schindelin *et al.*, 2012). Bands representing the different proteins were quantified and normalised against loading control. All the biological replicates for each experiment were normalised against the average of wild type or controls.

2.6.3 Solubility assay

This biochemical assay aims to examine the solubility of a given protein. In this study, this analysis was used to check whether ALS linked variants can cause altered solubility of the protein of interest. This assay can be carried out with a range of detergents that have different protein solubilisation abilities. In this study I use the RIPA/Urea based assay, which has a moderate level of solubility.

Transfected HEK293T cells were harvested using PBS complemented with proteinase inhibitors (Pi) and phosphatase inhibitors (Pho-STOP), they were spun for 30 seconds at 10,000 g at 4ºC, the supernatant was removed and the cells were resuspended in 200 µl of RIPA buffer (150mM NaCl, 1% triton, 0.1% SDS, 0.5% DOC (2,5-Dimethoxy-4-chloroamphetamine), 20mM Tris (pH 7.4), proteinase inhibitors (1 tablet per 50mL) and phosphatase inhibitors (PhoSTOP; 1 tablet per 50mL)). The cells were left on ice for 10 minutes and sonicated briefly, 20 µl of lysate was mixed with 20 µl of 2X lysis buffer (described in section 2.6.2.1), boiled for 10 minutes and stored at -20 ºC. The rest of the

lysate was spun down at 21,293g in 4ºC for 30 minutes, this step separated the insoluble fraction (pellet) from the soluble fraction (supernatant). 60 µl of the supernatant were mixed with 60 µl of 2X lysis buffer, boiled for 10 minutes and stored at -20 ºC. The rest of the supernatant was discarded and the pellet was washed with 1 ml of RIPA buffer twice. Samples were then spun at 21,293g in 4ºC for 30 minutes and the supernatant discarded again. Pellets were then resuspended in 10 µl of urea buffer (7M Urea, 2M Thiourea, 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 30mM Tri-HCL pH 8.5, stored at -20ºC) and shaken for 30 minutes at room temperature to dissolve the insoluble fraction. 10 µl of 2X lysis buffer were added to the insoluble fraction and the samples were stored at -20ºC.

The samples were analysed by western blotting as described in section 2.6.2. Lysate fractions were quantified as described in section 2.6.2.1 and an equal amount of the three fraction per sample was loaded on the gel. Prior loading the lysate and the soluble fractions were boiled for 10 minutes and the insoluble fraction was centrifuged for 30 minutes at 4 ºC at 21,293g to avoid loading debris on the gel.

All the fractions were quantified in relation to the lysate and, when possible, normalised against a loading control.

2.6.4 Nuclear/cytoplasmic fractionation

This assay analyses the ratio of protein contained in the nucleus against the amount of protein localised the cytoplasm. This analysis was used to verify whether ALS linked variants, found in fALS patients, altered this ratio.

Transfected HEK293T cells were harvested using PBS complemented with Pi and Pho-STOP, they were spun for 30 seconds at 10,000 g at 4ºC, supernatant was removed and the pellets were resuspended in 70 µl of HBL buffer (10mM Tris pH 7.4, 10mM NaCl, 3mM $MgCl_2$, 21mM EGTA ((ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), 1mM EDTA, proteinase inhibitors) and left on ice for 10 minutes. During this time cells were lysed using a 1 ml syringe (Fisher) with a 25G needle (Fisher) 10 times, which

would lyse the outer membrane of the cells leaving the nucleus intact. 20 µl of the lysate were then mixed with 20 µl of lysis buffer (described in section 2.6.2.1) and stored at -20ºC. The rest of the lysate was spun down for 5 minutes at 4ºC at 978g. 40 µl of the supernatant were mixed with 40 µl of lysis buffer and stored at -20ºC. All the supernatant was removed and the pellets were washed in HBL buffer complemented with 1% NP-40 (Calbiochem) twice, resuspended in 50 µl of RIPA buffer (described in section 2.6.3), briefly sonicated and stored at -20ºC.

Samples were then quantified as illustrated in section 2.6.2.1, diluted in loading buffer (described in section 2.6.2.2) and analysed by western blot as described in section 2.6.2.

All the fractions were quantified in relation to the lysate and normalised against a loading control.

2.6.5 Immunoprecipitation (IP) assay

This technique allows the precipitation of a particular protein with its binding partners through the specific binding of the protein to an antibody. This reveals which proteins interact with the protein of interest. In this study this technique was used to verify whether the binding of the protein of interest with a specific binding partner was altered by the integration of ALS linked variants. In this study IP were carried on with HA tagged proteins.

Transfected (or co-transfected) HEK293T cells were harvested using PBS complemented with Pi and Pho-STOP, they were spun for 30 seconds at 10,000 g at 4ºC, supernatant was removed and the pellets were then resuspended in 150 µl of IP buffer (50mM tris pH 7.4, 150 mM NaCl, 1% triton X-100, proteinase inhibitors). Samples were left on ice for 10 minutes and then centrifuged at 21,293g in 4ºC for 30 seconds. Pellets were discarded and the supernatant transferred in a different tube. 20 µl were taken from each sample and mixed with 2x loading buffer (described in section 2.6.2.2) complemented with 1M Dithiothreitol (DTT, Sigma) in a 1:4 ratio (i.e. 200 µl of 1M DTT in 800 µl of loading buffer), boiled for 10 minutes and stored at -20 ºC. The rest of the

lysate was then “pre-cleaned” by incubation with 20 µl of Dynabeads Protein G for Immunoprecipitation (Life Technologies, 10003D) for 2 hours at 4ºC to avoid any specific binding of the protein of interest with the magnetic beads. Following the pre-cleaning phase, beads were separated from the lysate using a magnet, 20 µl from each sample were mixed with 2x loading buffer complemented with DTT, boiled for 10 minutes and stored at -20 ºC. The remaining lysate was then transferred to a new tube where the antibody for the protein of interest was added at the appropriate concentration (in this study rabbit antibody against HA tag was used at a concentration of 1:100). 20 µl of Dynabeads Protein G were added to the mix and the samples were left to roll at room temperature for 2 hours. In this time beads would bind to the antibody, which would bind to the protein of interest, obtaining its precipitation. After this time beads were separated from the flow through using a magnet and 20 µl from each sample (Flow through, FT) were mixed with 2x loading buffer complemented with DTT, boiled for 10 minutes and stored at -20 ºC. The rest of the supernatant was discarded. The beads were then washed six times with 500 µl of IP buffer each time, resuspended in 20 µl of 2x loading buffer complemented with DTT and boiled at 100 ºC for 10 minutes.

IP processed samples were not quantified. 4 µl of lysate (and flow through when possible) and 9 µl of IP fraction were loaded on the gel and analysed by wester blotting as described in section 2.6.2.

2.6.6 Phosphorylation assay

Cells were harvested with PBS complemented with proteinase inhibitors spun down as described in section 2.6.2.1 and resuspended in RIPA buffer (see section 2.6.3) complemented with proteinase inhibitors. The amount of protein was quantified as explained in section 2.6.2.1 and 6 µg of protein per sample was put in a new tube. Samples were then sonicated briefly and processed as described in protein dephosphorylation protocol provided by abcam (http://www.abcam.com/protocols/protein-dephosphorylation-protocol). Briefly, they were incubated at 37 °C for 30 minutes with 3µl of 1X CIP buffer (100 mM NaCl, 50 mM Tris-

HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH to 7.9 at 25ºC) complemented with proteinase inhibitors and 3μl of alkaline phosphatase (Roche). As a control all the samples had an untreated duplicate that was processed identically only without adding the alkaline phosphatase. Samples analysed by western blotting as described in section 2.6.2 with the difference that the samples were ran on a NuPAGE Novex 3-8% Bis-Tris Midi gels.

2.6.7 Non denaturing (or native) gel

This technique allows the analysis, discerning on the size and the antibody labelling, of the interaction of the protein with itself, molecular mass estimations, and assessment of the purity of native proteins. In this study, this technique was used to verify the ability of TBK1 to homo dimerise when mutated compared to the wild type form.

In this technique transfected HEK293T cells were harvested with PBS complemented with Pis and PHOstop centrifuged at 30 seconds at 10,000 g at 4ºC. Samples were then processed using The NativePAGE™ Novex® Bis-Tris Gel system (Thermo Fisher Scientific) following manufacturer's instructions. This system uses Coomassie G-250 instead of SDS, which confers the protein a negative charge so that they can migrate towards the anode during electrophoresis, but it does not disrupt their binding or folding. Briefly, samples (24 μl) were lysed using 4X NativePAGE™ Sample Buffer (8μl, Thermo Fisher Scientific) and pipetting samples up and down. Cells were then centrifuged at 20,000 g for 30 minutes at 4°C and the supernatant was loaded in a NuPAGE Novex 4-12% Bis-Tris Protein Gels (1.5mm; 10 well, Thermo Fisher Scientific) alongside NativeMark™ Unstained Protein Standard (Thermo Fisher Scientific). As a running buffer 1X NativePAGE™ Anode Buffer was used for the externa chambers of the running tank, prepared according manufacutrer's instructions. 1X NativePAGE™ Cathode Buffer (light blue) was used for the internal chamber prepared according to manufacturer's instructions. The gel was run at 150 V for 2 hours and transferred using wet transferred method. The gel was removed from its plastic case and placed over a Polyvinylidene difluoride (PVDF) membrane, which was pre-activated with methanol for 30 seconds. On either side of the PVDF membrane and the gel, filter paper or 4 layers of sponge

previously wetted in transfer buffer (50 ml of NuPAGE® Transfer Buffer (20X, Life Technologies), 20% methanol, ultra-pure water to 1 litre). This was placed in a transfer tank which was filled with transfer buffer. The transfer reaction was operated at 300 mA for 1h in ice. Subsequently, the PVDF membrane was incubated with 20 ml of 8% acetic acid (Sigma) for 15 minutes to fix the proteins on the membrane. This was then washed with ultra-pure water, air dried and rinsed with methanol to remove the stains gained in the transfer stage. The membrane was then washed in ultra-pure water, incubated with 5% BSA as described in section 2.6.2.3 and analysed with immunoblotting as described in section 2.6.2.4.

2.6.8 Membrane potential assay

This analysis was carried out using the NucleoCounter NC-3000 (Chemotec) and was employed to detect disruption of mitochondrial membrane potential in individual cells. This is known to precede apoptosis and chemical-hypoxia-induced necrosis as well as being an index of the level of mitochondrial damage. This technique uses a lipophilic cationic dye (JC-1) that shows a potential dependent accumulation in mitochondria. In healthy cells, mitochondrial membranes will be negatively charged and this will cause the accumulation of JC-1 in the mitochondrial matrix. When JC-1 accumulates in the mitochondria, this forms precipitate which will become red fluorescent. In apoptotic cells, the mitochondria potential collapses and JC-1 will remain green fluorescent in the cytoplasm.

In this study, this technique was only used to analyse primary fibroblasts. About 100,000 cells were harvested per sample as described in section 2.5.4. Pellets were then resuspended in 100 μl of PBS, 1.25 μl of Solution 7 (Chemotech), containing the JC-1 dye, were added to each sample and incubated for 30 minutes at 37 °C. The stained cells were then centrifuged at 400 g for 5 minutes and the supernatant discarded. The pellet was then washed with 1 ml of PBS twice and resuspended in 20 μl of PBS complemented with 1μg/ml DAPI, this will penetrate only dead cells, which will be

excluded from the analysis. 10 µl of the resuspended cells were then loaded on a NC-slide A8 (Chemotec) and ran on the nucelocounter using the "Mitochondrial Potential Assay" protocol. As a positive control I used a control fibroblast line treated with 100 µM FCCP for 30 minutes. The analysis was conducted using the positive control as a reference to establish an unbiased gating criteria.

2.6.9 Cell mitochondria stress test

This assay was carried out using the Seahorse XFe96 Analyser (Agilent), this machine allows the measurement of oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) in a 96 well plate format. These two parameters are used to compare the metabolic state of the cells, indicating the level of mitochondria respiration and glycolysis activity, respectively.

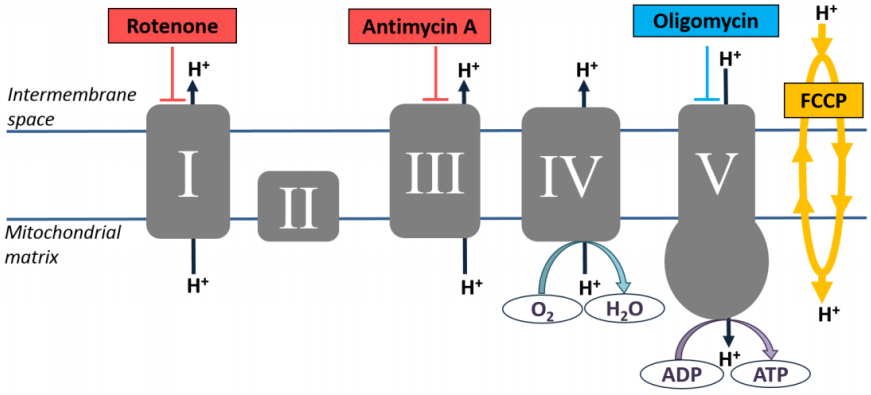


Figure 2.7 - Diagram of the effect of the used compounds in the electron transport chain (Agilent).

In this study I used the Agilent Seahorse XF Cell Mito Stress Test (Agilent), which indicates important parameters of mitochondrial function by measuring the OCR of the cells. The assay uses three sequential drug injections (oligomycin, FCCP, and a mix of rotenone and antimycin A) and it measures basal respiration, ATP production, maximal respiration, and non-mitochondrial respiration at different time points (as illustrated in Figure 2.7). Proton leak and spare respiratory capacity are then calculated using these parameters. This test was carried out using the Agilent Seahorse XF Cell Mito Stress Kit

(Agilent) following manufacturer's instructions. Briefly, primary fibroblasts were plated in the provided 96 well plate 48 hours before the day of the test at a density of 10,000 cells/well. The day prior to the assay the machine was switched on and the sensor cartridge was left hydrating in Seahorse XF Calibrant (Agilent) in an incubator at 37 °C with no CO_2. On the day of the test the medium was prepared by supplementing Seahorse XF Base Medium (Agilent) with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose, warming it up to 37 °C and adjusting the pH to 7.4. The compounds were then prepared at the desired stock concentrations and loaded on the cartridge at the concentration needed (oligomycin 2 μM, FCCP 1μM, Rotenone/Antimycin A 0.5 μM). The cartridge was then loaded on the machine, which was left calibrating while the plated cells were kept at 37 °C with no CO_2 for 45 minutes. After the incubation time, the plate was loaded on the machine and the Agilent Seahorse XF Cell Mito Stress Test was started.

The data obtained from this analysis was downloaded and analysed in GraphPad (Prism) format. Calculations were obtained using Agilent guidelines, as illustrated in Figure 2.8.

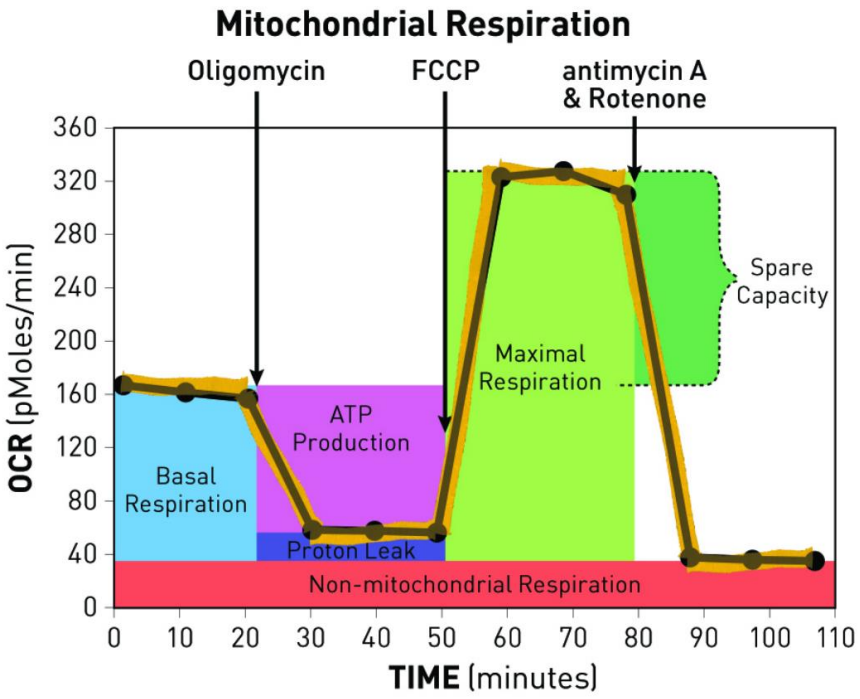


Figure 2.8 - Profile of key parameters of mitochondrial respiration (Agilent). OCR=oxygen consumption rate.

2.7 Statistical analysis

All statistical analysis was carried out using GraphPad (Prism 7.02). p values were considered significant when <0.05. In particular: * refers to p < 0.05, ** refers to a p<0.01, ***refers to p<0.001 and **** refers to p< 0.0001. Different statistical test were applied on different sets of data. All statistical tests used in this study are explained below:

- A t-test was used to compare the mean of two groups of data. Unless otherwise stated, t tests were unpaired, two tailed with 95% confidence intervals. A multiple t-test using Holm Sidak method (alpha=0.05) was used to compare two groups composed by two sets of data.
- Fisher's test and Chi square tests were used to analyse categorical variables. Chi square followed by Yate's correction was used on variables of large value.
- One way ANOVA was used to compare the mean of three or more sets of data. This test was adjusted by Dunnett's correction when means were compared with one

control mean, whereas when every mean was compared with every other mean Tukey's correction was used.

- When three or more groups of data were analysed by two factors a two way ANOVA test followed by Sidak correction was applied.

Chapter 3 – Genetic and functional characterisation of TBK1 ALS-associated variants

3.1 Background

In March 2015, heterozygous loss of function variants in TANK-binding kinase 1 (*TBK1*) were found to be significantly enriched in ALS patients against control by two independent studies (Cirulli et al. 2015; Freischmidt et al. 2015). Following this discovery, over 100 TBK1 variants have been identified in ALS and FTD patients (Appendix B), making TBK1 mutations the third or fourth most frequent cause of ALS and FTD, respectively, in populations of European ancestry (Freischmidt *et al.*, 2016).

TBK1 encodes for a ubiquitously expressed 729 amino acid protein kinase, TBK1, which is involved in many pathways such interferon (IFN) and pro-inflamatory cytokine production in the defence against pathogens (Fitzgerald *et al.*, 2003), autophagic digestion of protein aggregates, organelles and pathogens (Thurston *et al.*, 2009; Wild *et al.*, 2011; Pilli *et al.*, 2012) and cell growth (Clément, Meloche and Servant, 2008) (Figure 3.1).

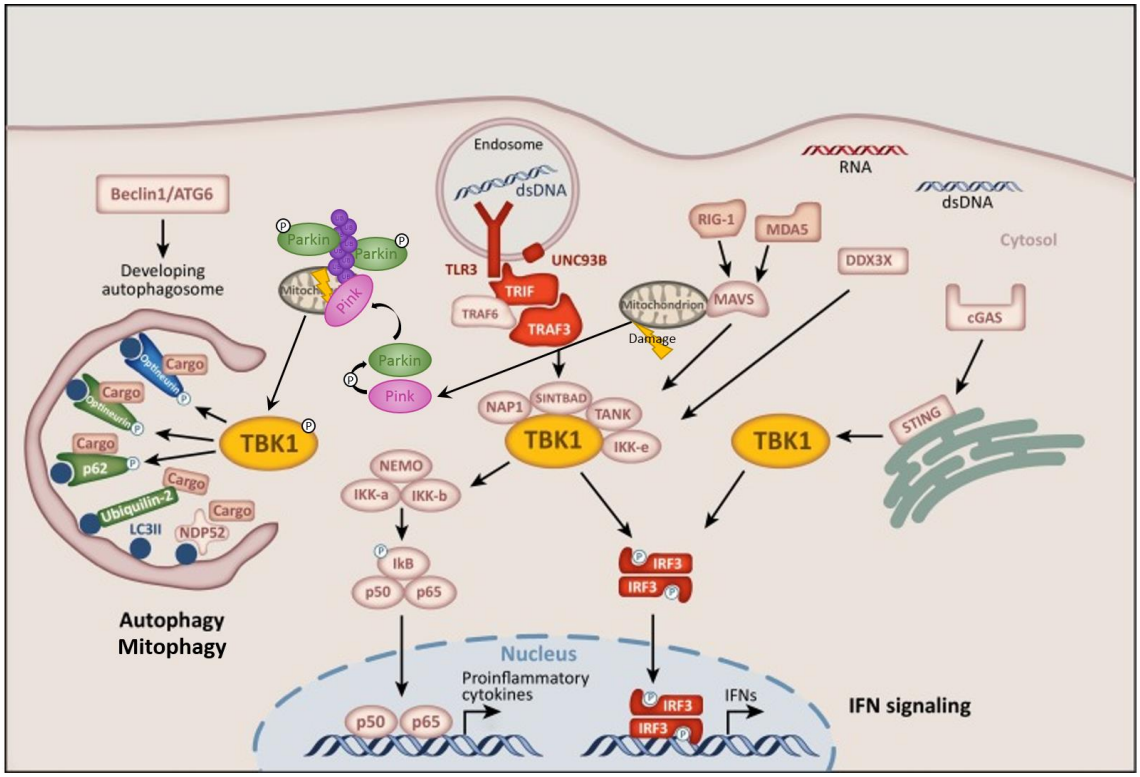


Figure 3.1 - TBK1 molecular pathways. From left to right: TBK1 involvement in autophagy, mitophagy, in the production of proinflammatory cytokines and IFN signalling (adapted from Markey et al. 2016)

3.1.1 An insight into TBK1 structure

TBK1 is composed by four domains: a kinase domain (KD), responsible for its kinetic activity, an ubiquitin-like domain (ULD), a scaffold dimerization domain (SDD) and a C-terminal domain (CTD), involved in TBK1 association with binding partners such as optineurin (OPTN), an important autophagy receptor (Tu et al., 2013) (Figure 3.4a,b). TBK1 has been shown to homo-dimerise through a central axis formed by the two SDD domains interacting with each other (Figure 3.4c). This structure is stabilised by the ULD and the KD that interact with each other and with the SDD axis, forming a globular head that stabilises the whole structure (Tu *et al.*, 2013). The linker region between the ULD and the SDD also contributes to the tertiary structure by interacting with the SDD axis. The interactions between the ULD, the SDD and their linker region are highly hydrophobic and prevent the homodimer from being dissociated when carrying out its functions. On the other hand, interactions of the KD within this structure are mainly polar (Tu *et al.*, 2013).

TBK1 activation has been demonstrated to be a multistep process that begins with the Lys-63-linked polyubiquitination, which is required for Ser172 phosphorylation within the activation loop. This causes a critical change in protein conformation promoting the active position of the C-helix in the SDD domain and facilitating the final step of homo-dimerisation, essential for mature kinase activity (Ma *et al.*, 2012; Tu *et al.*, 2013).

3.1.2 TBK1 in inflammatory pathways

In the inflammatory pathways, TBK1 is activated by toll like receptor 3 (TLR3), which is, in turn, activated by double strand RNA (dsRNA), mainly carried by retroviruses. Toll like receptors (TLRs) are a family of proteins that play an important role in pathogen recognition and activation of innate immunity (Markey *et al.*, 2015). TLR3 activates TBK1 through the recruitment of its adaptor TRIF (TIR-domain-containing adaptor-inducing interferon-β). Active TBK1 will, in turn, phosphorylate Interferon Regulatory Factor 3 (IRF3) which will homodimerise and migrate to the nucleus, where it will carry out the transcription of antiviral type I and III interferons (IFNα/β/λ) (Fitzgerald *et al.*, 2003; Sharma, 2003; Yamamoto, 2003). TBK1 can also be activated by cytoplasmic factors such as RIG-I-like receptors (RLRs), activated by viral RNA (Ishii *et al.*, 2006; Paz *et al.*, 2006; Loo and Gale, 2011), or cytoplasmic DNA receptors activated by double strand DNA (dsDNA) (Sun *et al.*, 2013). TBK1 activation by these factors also results in the phosphorylation of IRF3 and, occasionally, IRF7, leading to the production of IFNα/β/λ (Ishikawa and Barber, 2008; Saitoh *et al.*, 2009).

It is not clear whether TBK1's role in the innate immune response is relevant for ALS pathogenesis. Interestingly, several recent studies have demonstrated that some ALS-associated *TBK1* variants abolish or reduce the ability of TBK1 to phosphorylate IRF3 (Freischmidt *et al.*, 2015; Kim *et al.*, 2016; Tsai *et al.*, 2016; Pozzi *et al.*, 2017). IRF3 levels were also found to be decreased in patient derived fibroblast harbouring p.I472Sfs *TBK1* variant (Kim *et al.*, 2016). Furthermore, two studies have shown that interferon beta (IFNβ) activation is diminished by some ALS-linked *TBK1* variants, when compared to wild type (Freischmidt *et al.*, 2015; Kim *et al.*, 2016).

3.1.3 TBK1 in autophagy

Autophagy is a homeostatic process conserved throughout evolution that induces the degradation of dysfunctional organelles, unnecessary or unwanted cellular proteins or pathogens in order to maintain healthy cell functions (Klionsky, 2007). This process takes place intracellularly, where bulk cargo (i.e. protein aggregates or dysfunctional organelles) are recruited in autophagic lysosomes for destruction or amino acid recycling (Figure 3.2). Autophagy is a tightly regulated and highly specific process which relies on the efficient organisation of a vast number of components, autophagy related proteins (ATGs) such as beclin-1 (ATG6), ATG16L, ATG12 and ATG5 (Klionsky, 2007; Fujita *et al.*, 2008; Klionsky and Schulman, 2014). A special category of proteins, called autophagy receptors, including OPTN, nuclear dot protein 52 kDa (NDP52) and p62, recognises ubiquitinated cargoes and binds them to the autophagic membrane through light chain 3 (LC3-II). LC3-II is found on the surface of the autophagophore, which engulfs and recycles the ubiquitinated cargoes into an autophagosome (Stolz, Ernst and Dikic, 2014) (Figure 3.1). The role of TBK1 in autophagy has been extensively studied in the context of *Salmonella* infections, where TBK1 is involved in the phosphorylation of OPTN and NDP52, which recognise ubiquitinated *Salmonella* and deliver it to the autophagosome (Morton *et al.*, 2008; Thurston *et al.*, 2009). This will, eventually, fuse with lysosomes to form a structure called autophagolysosome that degrades and recycles its contents (Figure 3.2). TBK1 is also thought to play a key role in the formation of the autophagolysosome (Pilli *et al.*, 2012).

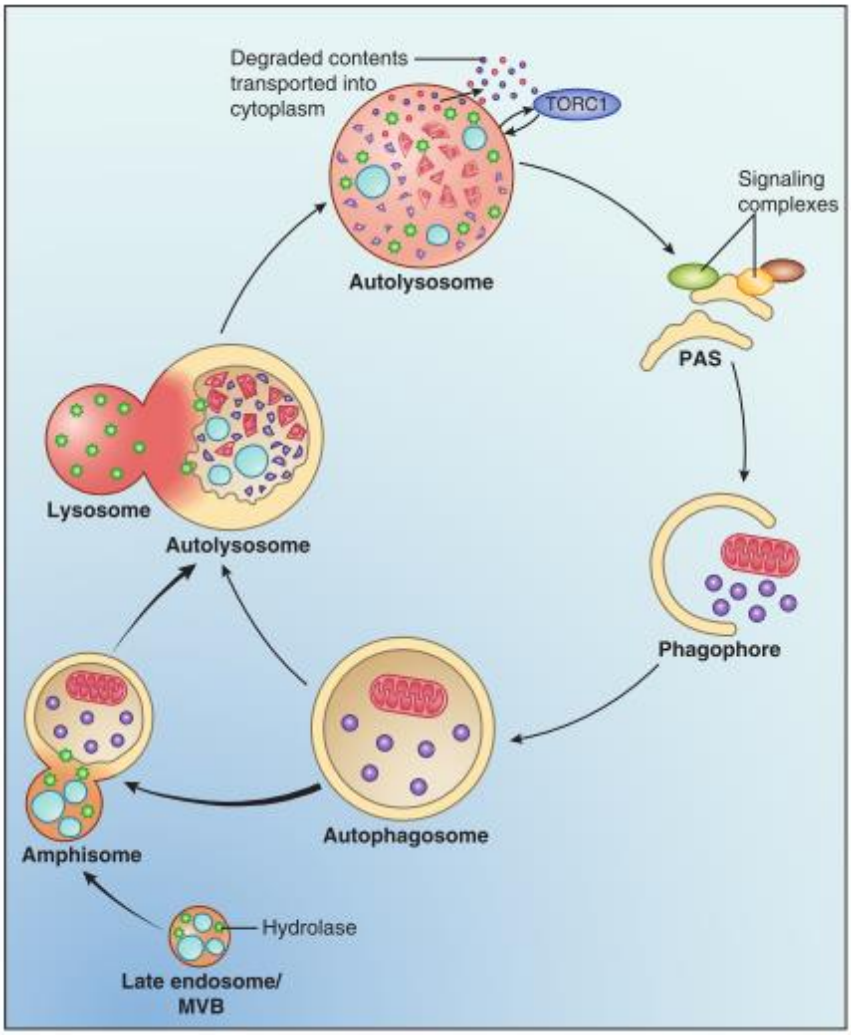


Figure 3.2 - Overview of the autolysosome formation. Starting from the highly regulated formation of the phagophore. Elongation of the membrane around the targets of autophagy and its closure to form the autophagosome, which fuses with a lysosome to form the autolysosome, where the degradation takes place. MVB, multivesicular body; PAS, preautophagosomal structure. (Adapted from Nixon 2013).

In the context of ALS, TBK1 has been found to co-localise with OPTN in SOD1 aggregates in HeLa cells and in a SOD1 transgenic murine model harbouring SOD1 p.G93A. Here, TBK1 is thought to phosphorylate OPTN on Ser177 which will deliver SOD1 protein aggregates to the autophagolysosome (Korac *et al.*, 2013).

Since TBK1 variants were reported in ALS, a few studies have looked at the interaction between TBK1 and OPTN and shown that some ALS-associated nonsense variants, or those mapping in the SDD domain (p.L59Ffs, p.R444X, p.Ile472Serfs*8, p.M559R, p.E696K, p.690-713del), affect TBK1 binding with OPTN (Freischmidt et al. 2015; Kim et al. 2016; Pozzi et al. 2017; Richter et al. 2016; Tsai et al. 2016). Further investigation of

how TBK1 is involved in the autophagy pathway and how disease associated TBK1 variants impact this mechanism is necessary in order to elucidate molecular mechanisms involved in these diseases.

3.1.3.1 TBK1 in mitophagy

TBK1 has recently been associated with a selective type of autophagy which specifically targets damaged mitochondria, mitophagy. This process was discovered in yeast (Kiššová *et al.*, 2004), whereby autophagy receptors recognise defective mitochondria and carry them to the autophagophore, where these receptors bind to LC3 through their LC3-interacting region (LIR) motif (Wild, McEwan and Dikic, 2014). One of the most studied pathways that regulates mitophagy is the PTEN-induced putative kinase 1 (PINK1)/Parkin mediated autophagy (Martinez-vicente, 2017). Disruption of this pathway has been linked to Parkinson's disease (PD), a neurodegenerative disease that selectively targets dopaminergic neurons (Kitada *et al.*, 1998; Valente, 2004). When mitochondria are damaged PINK1 accumulates in the outer mitochondrial membrane (OMM) where it recruits and activates Parkin, inducing its ubiquitin ligase activity (Matsuda *et al.*, 2010; Narendra *et al.*, 2010; Shiba-Fukushima *et al.*, 2014). Parkin successively ubiquitinates many proteins in the OMM (Geisler *et al.*, 2010; Chan *et al.*, 2011), these ubiquitinated proteins can then be recognised by autophagy receptors such as OPTN (Wong and Holzbaur, 2014; Calcagno *et al.*, 2016) and p62/SQSTM1 (Geisler *et al.*, 2010), which will guide the defective mitochondria to the autophagophore as described above. The binding of these cargo proteins to the autophagophore depends upon activation by TBK1, which is co-recruited together with OPTN (Matsumoto *et al.*, 2015; Moore and Holzbaur, 2016). Li and colleagues have hypothesised a model where both OPTN and TBK1 homodimerise and then associate in a heterotetramer, which is stabilised by the interaction of TBK1 CTD (residues 677–729) with OPTN n-terminal domain (NTD, residues 26–119). The hetero-tetrameric complex recognises the ubiquitin chains assembled by Parkin. TBK1 can now phosphorylate OPTN on Ser177 (Wild *et al.*,

2011) and Ser473 (Richter et al. 2016), which will proceed in the mitophagy pathway (Li *et al.*, 2016).

3.1.4 TBK1 in ALS and FTD

TBK1 was associated with ALS for the first time in 2015 when a WES study found an enrichment of TBK1 loss of function variants in 2869 ALS patients when compared to 6405 controls (Cirulli et al. 2015). This finding was confirmed by an independent study which identified a TBK1 mutation segregating with disease in a large family and an enrichment of TBK1 variants in 252 fALS. This study was the first to conduct functional characterisation of TBK1 mutations and showed that a subset of TBK1 mutations impaired IRF3 phosphorylation and/or OPTN binding.

Since then over 100 variants have been identified in ALS, ALS-FTD and FTD patients (Appendix B), however only seven studies have undertaken functional characterisation of any variants and the mechanisms underlying mutant TBK1 pathogenicity are still unclear (Gijselinck *et al.*, 2015; Pottier *et al.*, 2015; Kim *et al.*, 2016; Tsai *et al.*, 2016; Pozzi *et al.*, 2017; van der Zee *et al.*, 2017). There is a general consensus that TBK1 truncation mutations cause a loss of function through haploinsufficiency, as hypothesised by Freischmidt and colleagues in 2015 (Freischmidt *et al.*, 2015). As illustrated in Figure 3.3, the most abundant types *TBK1* ALS-associated variants are missense variants. The pathogenicity of these variants is often determined by co-segregation and in silico predictions (Freischmidt *et al.*, 2016), however, these tools are not accurate, and functional characterisation is necessary in order to determine the pathogenicity of each singular variant. One study has found that some *TBK1* missense mutations, including p.K401E and p.E969K, result in a lower expression of TBK1, leading once again to the haploinsufficiency hypothesis (Pottier *et al.*, 2015). However this does not seem to be true for every missense mutation (Freischmidt *et al.*, 2016; Kim *et al.*, 2016). Overexpression of some missense variants has shown that these fail to phopshorylate/bind IRF3 (i.e. p.R47H, p.D118N, p.R308Q, p.R357Q and p.M559R) or bind OPTN (i.e. p.M559R, p.E696K) (Freischmidt *et al.*, 2015; Pozzi *et al.*, 2017). This

suggests that missense mutations might be causing ALS and FTD through a loss of function mechanism.

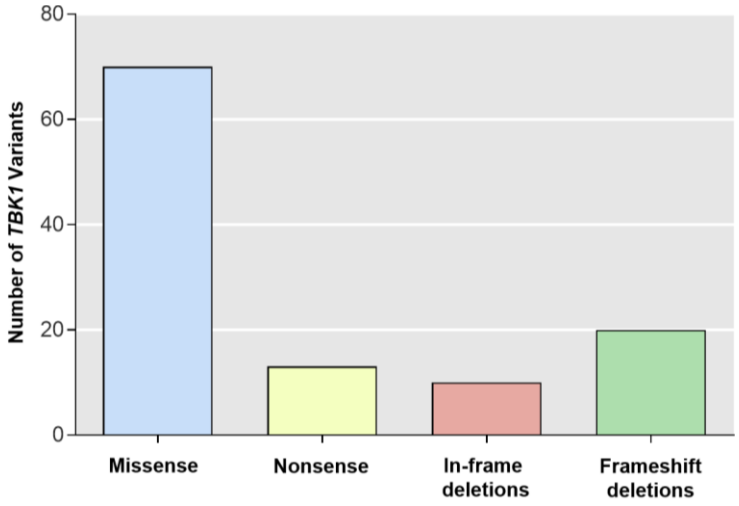


Figure 3.3 – Number of TBK1 variants found in ALS and FTD patients divided by subtype. For more details and references all variants are listed in Appendix B

Three post mortem studies have reported neuron loss, TDP-43 proteinopathy as well as p62 inclusions in post mortem brain tissue of patients harbouring TBK1 mutations (p.Gly272_Thr331del, p.Thr79del, Val97Phefs*2, p.Ala417Ter and p.Arg117Ter together with OPTN p.Gly538Glufs*27) (Gijselinck *et al.*, 2015; Pottier *et al.*, 2015; van der Zee *et al.*, 2017). The current hypothesis is that loss of TBK1 function impairs autophagy leading to the accumulation of TDP-43 aggregates, a hallmark of ALS.

	No of patients (variants)	Clinical presentation			M/F	Mean age of onset ±SD (years)		
		ALS (fALS)	ALS-FTD	FTD		M+F	M	F
All	110 (80)	57 (17)	16	19	45/39	60.4±10.9	58.1±11.3	62.0±10
Loss of function	59 (32)	30 (13)	10	12	30/24	62.5±9.7	61.0±9.6	64.3±9.7

Table 3.1 - Summary of the clinical data available in the literature. Adapted from Pozzi et al. 2017.

3.1.5 TBK1 in other pathologies

Heterozygous missense mutations in TBK1 have been found in patients with herpes simplex encephalitis (HSE), a neurological condition caused by rare complication of

herpes simplex virus-1 (HSV1) infection (Whitley and Gnann, 2002). It is believed that these complication might arise in the case of an impaired TLR3-IFN pathway which can be caused by mutations in *TBK1* (Zhang *et al.*, 2013). It is not clear how TBK1 causes HSE and both dominant negative and haploinsufficiency mechanisms have been hypothesised (Herman *et al.*, 2012).

TBK1 has also been linked to glaucoma, a neurodegenerative disease that affects the retinal ganglion cells in the optic nerve that is also linked to OPTN mutations (Quigley, 2011). More specifically, an increased number of copies of TBK1 has been reported in patients affected by normal tension glaucoma (NTG) (Ritch *et al.*, 2014; Awadalla *et al.*, 2015). Interestingly, in this case the disease is thought to be caused by a gain of function (Fingert *et al.*, 2011), presenting a different disease mechanism from ALS.

3.1.6 TBK1 mouse models

TBK1 *in vivo* characterisation has been particularly challenging. TBK1 is highly conserved in mammals (Tojima *et al.*, 2000) and homozygous deletion of *TBK1* in mice is lethal early on in the embryonic stage due to apoptosis and hepatic tissue loss (Bonnard *et al.*, 2000). However, a mouse model expressing a truncated form of TBK1 (devoid of exon 2, in the kinase domain) in homozygosity, survives the embryonic phase and shows a reduced levels of TBK1 protein, with no kinase activity. Macrophages from these mice have shown reduced IRF3 DNA binding property and IFNβ response, however, there was no mention of any neurological phenotype or autophagy deficits (Marchlik *et al.*, 2010).

3.2 Aim of this project

The aim of this project was to identify and functionally characterise a subset of protein changing and potentially damaging *TBK1* variants, identified in a cohort of 699 fALS cases. The ultimate aim of the project was to contribute to the clarification of TBK1 role in the pathogenesis of ALS.

3.3 Material and methods

Cell culture and molecular and biochemical assays have been carried out as described in Chapter 2. Techniques specific to this chapter are explained below.

3.3.1 Nucleotide variant and significance analysis

This analysis was carried out by the Shaw lab bioinformatician Simon Topp. To assess the relative abundance of *TBK1* variants in our patients, a burden test was performed using ExAC database as a control cohort. In brief, significance was assessed in the burden test using a Fisher's Exact, two tailed statistic calculated on the number of fALS and ExAC Non-Finnish European (NFE) variants remaining after annotation and filtering by the criteria described in section 2.2.

Common ancestry between samples was taken from existing familial annotation, where available, and also deduced from inheritance by descent (IBD) analysis in vcftools (Yang *et al.*, 2011) and King (Manichaikul *et al.*, 2010), using only variant positions covered to a depth >10 in >85% of fALS cases, and recoding all missing data to a heterozygous reference genotype (0/0).

3.3.2 PCR amplification and sequencing of c.992+1G<A (p.G272_T331del)

RNA was extracted and cDNA synthesised from the lymphoblastoid cell lines derived from the patient carrying the c.992+1G<A (p.G272_T331del) mutation and from a healthy control, as described in section 2.5.5. The whole *TBK1* gene was then amplified using primers located in the first and last exons to amplify the entire open reading frame of the transcript. The product was sequenced using the forward and reverse primers flanking exon 8 (Table 3.2).

	Forward	Reverse
Amplification	ATGCAGAGCACTTCTAATCATCTGTGGC	CTAAAGACAGTCAACGTTGCGAAG
Sequencing	TTGAAGGGCCTCGTAGGAAT	TCAGCCATCGTATCCCCTTT

Table 3.2 – Primers used for amplification and genotyping of c.992+1G<A (p.G272_T331del). Primers were designed using Primer 3 as described in section 2.3.1. Sequences are represented 5' – 3'.

3.4 Results

3.4.1 No *TBK1* variants are present in *TBK1* variant hotspots in 174 sALS cases

In order to determine whether the variants identified in the exome sequencing project were also found and possibly replicated in a cohort of 174 apparently sporadic cases, I Sanger sequenced exon 2, 3, 5, 6, 7, 8, 9, 12 and 18. This was also conducted to identify any novel *TBK1* mutations. When this project was initiated in 2015, these were considered hotspots for *TBK1* ALS-linked mutations. The replication of the same variant in familial and apparently sporadic ALS cases is considered further evidence of the variant pathogenicity, however, no variants were identified in this cohort.

3.4.2 Whole Exome Sequencing in a fALS cohort detects 16 *TBK1* variants

Exome sequencing had been performed by previous lab members on 699 index cases from a cohort of fALS from eleven countries (UK, USA, Italy, Spain, Canada, Germany, Netherlands, Belgium, New Zealand, Israel and Ireland) (Table 2.1). These patients were negative for mutations in other known ALS genes including: *SOD1*, *TDP43*, *C9ORF72*, *FUS*, *PFN1*, *UBQLN2*, *OPTN*, *VCP*, and *ANG*, and the intronic *C9ORF72* repeat expansion. The DNA extraction and exome capture were performed by previous lab members Athina Soraya Gkazi and Jack Miller and sequencing was outsourced mainly to the Genomic Core Facility at Guy's Hospital.

Exome sequence data analysis described below was performed by Simon Topp who identified sixteen potentially deleterious protein-changing variants in *TBK1*, which were novel, or had an ExAC NFE carrier frequency of <1:20,000 individuals (Table 3.3). In concordance with results from other published ALS studies, this showed an abundance of potentially protein-changing *TBK1* variants in our familial cohort ($p=1.02e^{-10}$, Fisher's two-tailed test). Thirteen of these variants were absent from the following databases: 1000 genomes, UK10K, Exome Variant Server (EVS), and ExAC databases (n > 72,000). Two variants (p.R357Q, p.C471Y) were found once and one variant (p.R358H) was found. The p.G217R variant is present in two Dutch cases predicted to be first degree relatives (King kinship coefficient=0.314, vcftools Ajk=0.451), and is their only shared novel variant in a gene or pathway previously linked to ALS. Interestingly, the variant p.R357X is also present in a single fALS case in the ALS data browser (ALSdb, http://alsdb.org/index.jsp), however, this individual is unlikely to be closely related to the p.R357X carriers identified in our sequencing as they do not share any other rare variants. Amongst the previously excluded fALS samples, another novel missense variant (p.Y394D) was identified in a patient who harboured the known pathogenic p.M337V *TARDP* mutation, which segregated in their affected sibling. However, the available exome data did not have sufficient coverage to determine if the sibling also shared the *TBK1* variant and DNA was not available to allow us to perform Sanger sequencing. Furthermore, the *TBK1* variant p.R358H was present in both first-degree relatives of a kindred, but both were also carriers for the particularly aggressive p.R521C *FUS* mutation.

By mapping *TBK1* missense variants separately from nonsense variants we observed a striking difference in their distribution (Figure 3.4). Whilst nonsense variants were spread across the whole protein, missense variants tended to cluster within the functional kinase (KD) and ubiquitin-like domain (ULD). Interestingly, the ULD has been reported to interact with both the SDD and KD and these interactions play a vital role in the correct folding and dimerization of TBK1 (Li *et al.*, 2012; Tu *et al.*, 2013).

From a clinical point of view, the patients analysed in this study display an age of onset of 55.8 and a ration of male to female of 8/8 with an average duration of the disease (when known) of 34.5 months (Table 3.3).

Type Of Variant	Exon	Nucleotide Variation[a]	Residue Change	Number of cases	Control Frequency	Gender	Clinical Diagnosis	Site of Onset	Age of Onset (years)	Disease duration (months)	Reference
Nonsense Variants	2	c.4C>T	p.Gln2Ter	1	0/82,513	F	ALS	S	60	-	(Cirulli et al. 2015; Gijselinck et al. 2015)
	9	c.1069C>T	p.Arg357Ter	1{1}	0/82,519	F	ALS	UL	76	-	(Cirulli et al. 2015)
	18	c.1869_1875del	p.Met623IlefsdTer9	1	0/82,359	F	ALS	-	43	20.4	-
	18	c.1887_1890del	p.Gln629HisfsTer4	1	0/82,445	F	ALS	-	-	-	-
In Frame Deletions	4	c.236_238delCAA	p.Thr79del	1	0/82,314	M	ALS	R	67.8	7.3	(van der Zee *et al.*, 2016)
	-	992+1 G>A	p.Gly272_Thr331del	1{1}	0/80,635	M	ALS	-	46	24	-
	18	c.1928_1930delAAG	p.Glu643del	1{1}	0/82,323	M	ALS	UL	64	48	(Cirulli et al. 2015; Freischmidt et al. 2015; Gijselinck et al. 2015; van der Zee et al. 2016)
Missense Variants	2	c.64A>C	p.Asn22His	1{1}	0/82,490	F	ALS	-	49	12	(Cirulli et al. 2015)
	2	c.92A>G	p.Thr31Ala	1	0/80,639	M	ALS	-	-	-	-
	5	c.385A>G	p.Asn129Asp	1	0/82599	M	ALS	L	68	-	(Cirulli et al. 2015)
	6	c.649G>A	p.Gly217Arg	1 (1*)	0/82,595	M,M	ALS	-	-	-	(Cirulli et al. 2015)
	9	c.1070G>A	p.Arg357Gln	1 {1}	1/82,519	M	ALS	LL	50	94	(Freischmidt *et al.*, 2015; Pozzi *et al.*, 2017)
	9	c.1073G>A	p.Arg358His	1(1*)	7/82519	-	-	-	-	-	-
	9	c.1180T>G	p.Tyr394Asp	1{1#}	0/82,080	F	ALS	B	40	-	(Cirulli et al. 2015)
	12	c.1412G>A	p.Cys471Tyr	1	1/79,400	F	ALS	-	-	-	(Cirulli et al. 2015)
	15	c.1694A>C	p.Gln565Pro	1 {1}	0/75,603	F	ALS	UL	50	36	(Cirulli et al. 2015)

Table 3.3 – TBK1 mutations identified in ALS patients by this study and their clinical phenotype. [a] Mutation nomenclature as recommended by the Human Genome Variation society www.hgvs.org, utilising +1 as the A of the initiator Met codon, translation start site. (*) affected relative from the present cohort. Cases number=699. The number in brackets ({}) represent the cases reported in ALSdb (http://alsdb.org/index.jsp). {#} represents duplicated sample found in ALSdb. S=spinal, UL=Upper Limb, R=respiratory, L=limb, LL=Lower Limb, B=bulbar.

3.4.3 Selection of subset of variants likely to be pathogenic

To focus our functional characterisation of *TBK1* variants identified in this cohort, a short-list of three variants from the sixteen found was chosen on the basis of the following criteria. The first selection criterion was based on familial segregation and replication between published studies. To date, only one TBK1 missense mutation segregating with disease within a family has been identified (van der Zee *et al.*, 2017). We have shown relatedness for two individuals in our exome cohort carrying the p.G217R variant that made it a priority to study. The p.R357X variant was also detected in a second individual in ALSdb database, which is a further evidence of pathogenicity. The second criterion considered was the proportion of algorithms which predicted the variant to be damaging, and the third criterion was the location of the variant in the functional domains of TBK1 based on the homodimer crystal structure (PDB 4IM0, Figure 3.4). Three variants all scored highly: p.G217R (located in the KD, shared by an affected relative, and predicted to be damaging by 17/20 applied algorithms), p.R357X (located in the ULD and found to remove the entire SDD) and p.C471Y (located in the SDD and potentially involved in TBK1 homodimerisation (Figure 3.4a,b)

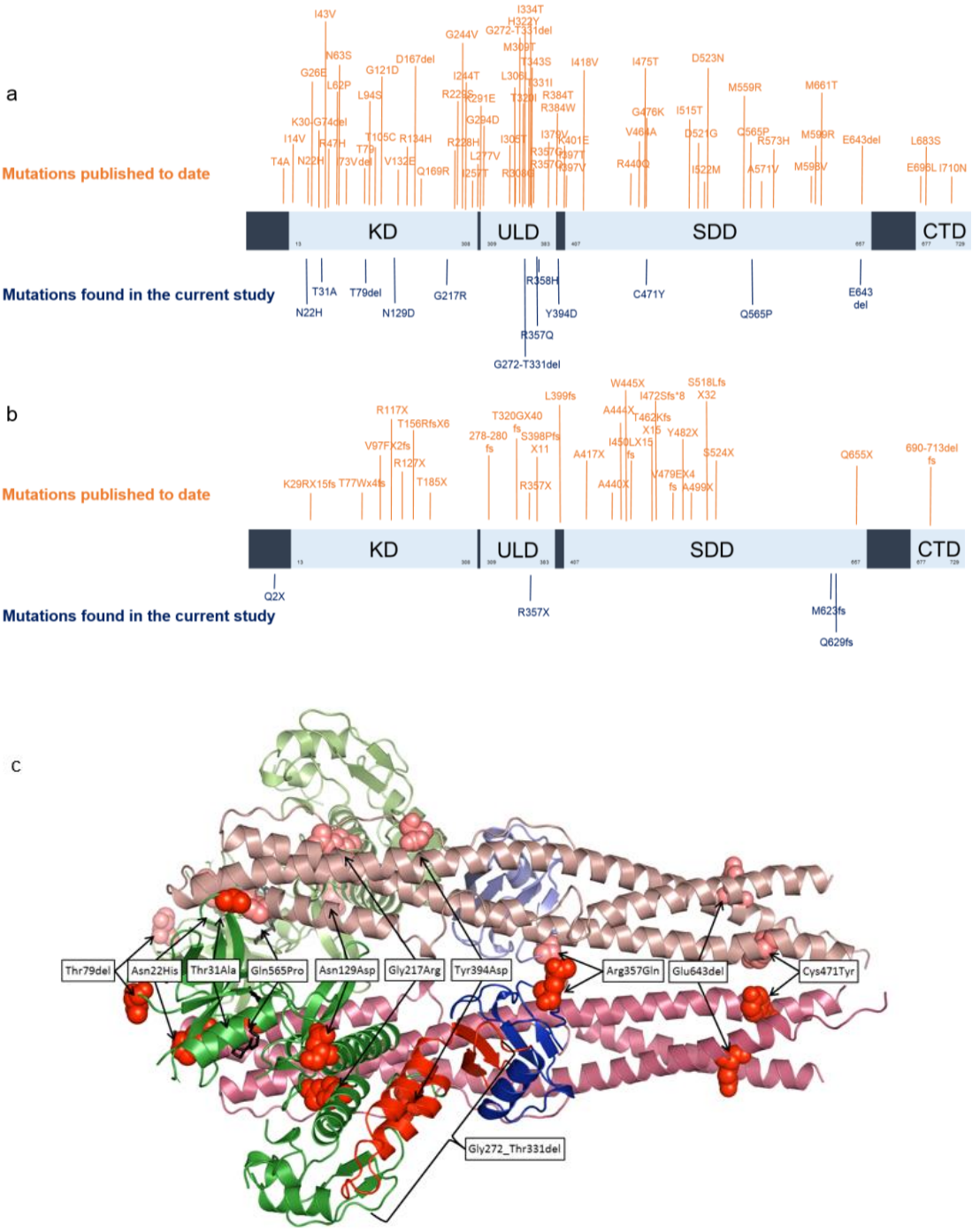


Figure 3.4 – TBK1 mutations identified to date and their location in TBK1 structure. (a,b) Schematic representation of TBK1 protein structure (Tu et al. 2013) showing a map of nonsense (a) and missense (b) variants found in literature and in our cohort. (KD = Kinase domain, ULD = Ubiquitin like domain, SDD = Scaffold dimerization domain, CTD = C-terminal domain). (c) TBK1 homodimer crystal structure (PDB 4IM0) mapping the mutations found in our study excluding premature stop codons and frameshift deletions. Figure generated by Simon Topp.

3.4.4 ALS-linked *TBK1* variants decrease the phosphorylation of the TBK1 target IRF3

Some ALS and FTD associated *TBK1* variants have previously been shown to diminish or abolish phosphorylation of the TBK1 target IRF3 (Freischmidt *et al.*, 2015; Kim *et al.*, 2016; Tsai *et al.*, 2016; Pozzi *et al.*, 2017). The efficiency of p.G217R, p.R357X and p.C471Y to phosphorylate IRF3 was therefore tested. HEK293T cells were transiently transfected with wild-type (WT) or mutant *TBK1* and IRF3 phosphorylation was quantified by western blot. The levels of total endogenous IRF3 were unchanged, but the level of phospho-IRF3 (pIRF3) as measured by western blot was significantly reduced in p.G217R and p.R357X variants compared to the WT (Figure 3.5a,b), however, the p.C471Y variant, which was predicted to be pathogenic by bioinformatics analyses, showed no difference from WT. Transfected HEK293T cells analysed for pIRF3 by immunocytochemistry confirmed that p.G217R and p.R357X abolished IRF3 phosphorylation, and, therefore, confirmed that these two mutations impair TBK1 kinase activity (Figure 3.5c).

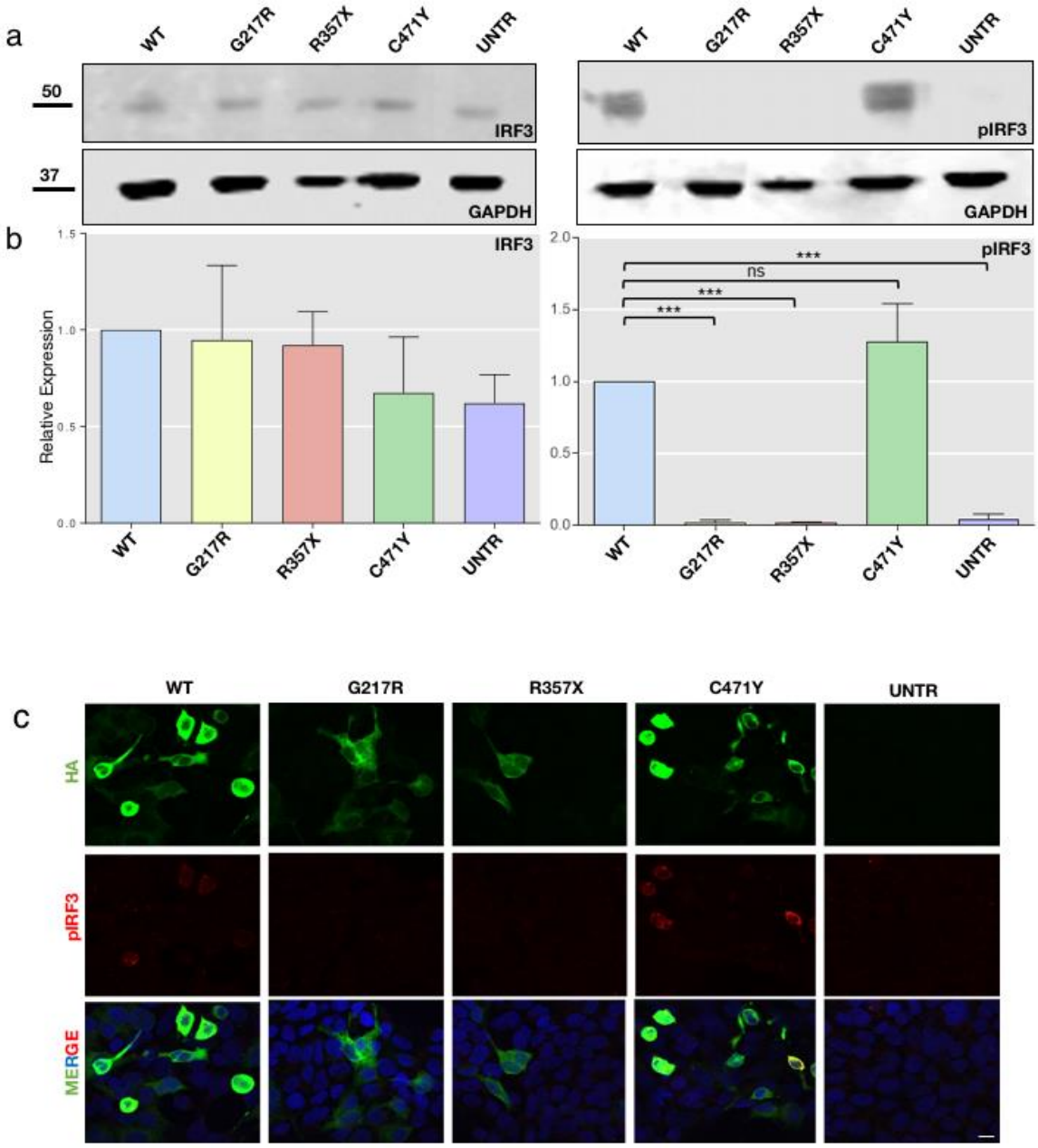


Figure 3.5 - TBK1 p.G217R and p.R357X impair TBK1 IRF3 phosphorylation. (a) Western Blot analysis of IRF3 (left) and pIRF3 (right). (b left) Quantitative analysis of blot in (a) left showing a similar expression level of endogenous IRF3 in cells (n=3 analysed by One Way ANOVA followed by Dunnet's correction p=0.5221). (b right) Quantitative analysis of blot in (a) right showing a significant decrease of expression of endogenous pIRF3 in cells transfected with TBK1-p.G217R and p.R357X (n=3, analysed by One Way ANOVA followed by Dunnet's correction p<0.0001). Error bars represent the standard error of the mean (SEM) (c) Qualitative immunocytochemistry of HEK293T transfected with TBK1-WT, p.G217R, p.R357X and probed for p-IRF3 confirming the result obtained by western blot analysis (scale bar = 10 µm)

3.4.5 ALS-linked *TBK1* variants decrease binding to and phosphorylation of OPTN

As described above, TBK1 is known to phosphorylate and regulate the activity of OPTN, a key receptor for poly-ubiquitinated proteins and mitochondria in the autophagy pathways (Richter et al. 2016). Co-immunoprecipitation (Co-IP) of HEK293T transiently co-transfected with Flag tagged OPTN and HA tagged WT, p.G217R, p.R357X and p.C471Y TBK1 was performed in order to determine whether these variants bound OPTN. HA tagged WT and p.C471Y consistently pulled down Flag tagged OPTN (Figure 3.6a). Interestingly, the same variants that impaired IRF3 phosphorylation (p.G217R and p.R357X) also dramatically reduced TBK1 binding to OPTN and its phosphorylation which occurred when WT and p.C471Y were co-transfected with OPTN. Phosphorylation was confirmed as the higher OPTN band, observed in WT and p.C471Y and absent for p.G217R or p.R357X, disappeared in the presence of alkaline phosphatase (Figure 3.6b).

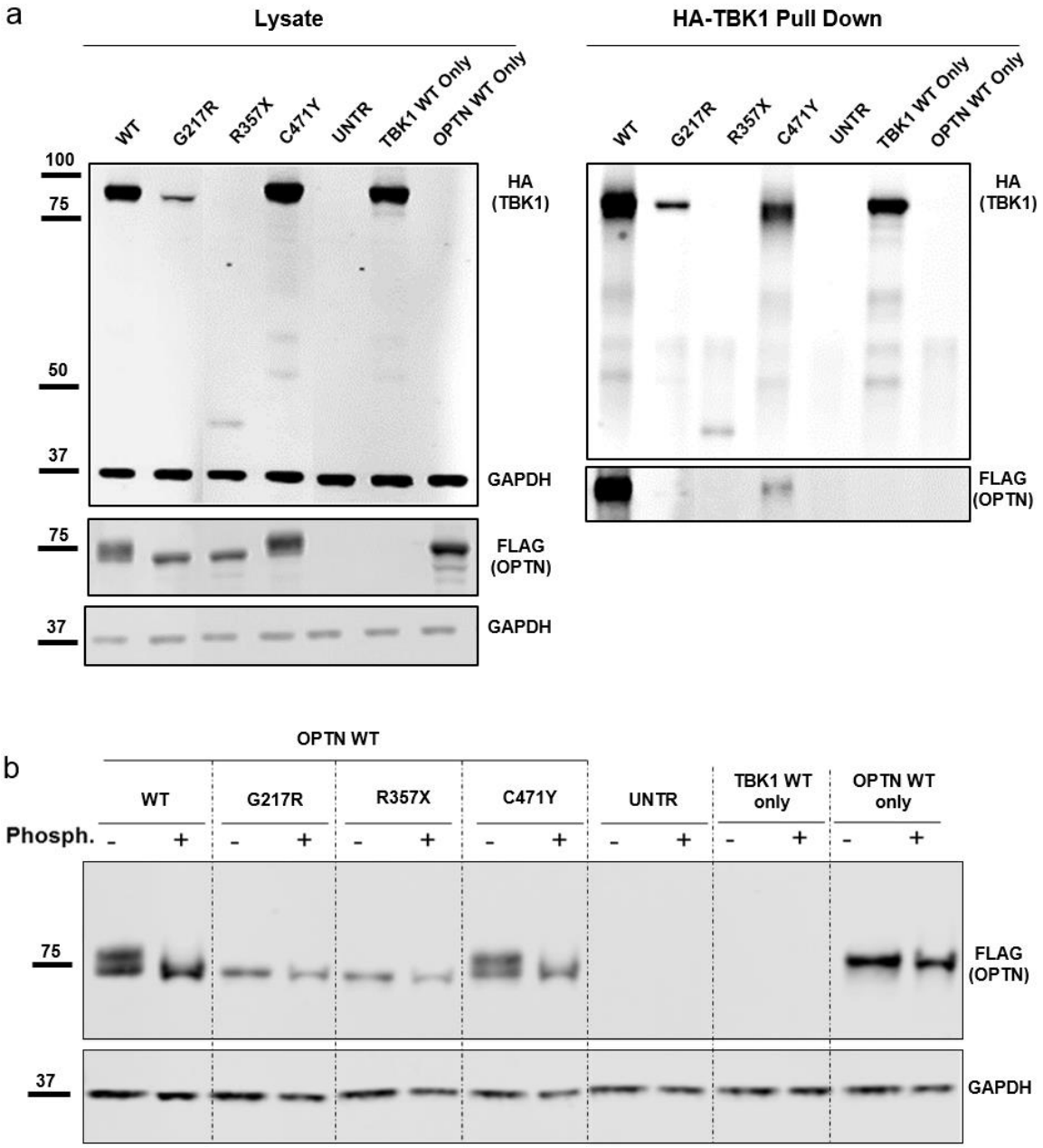
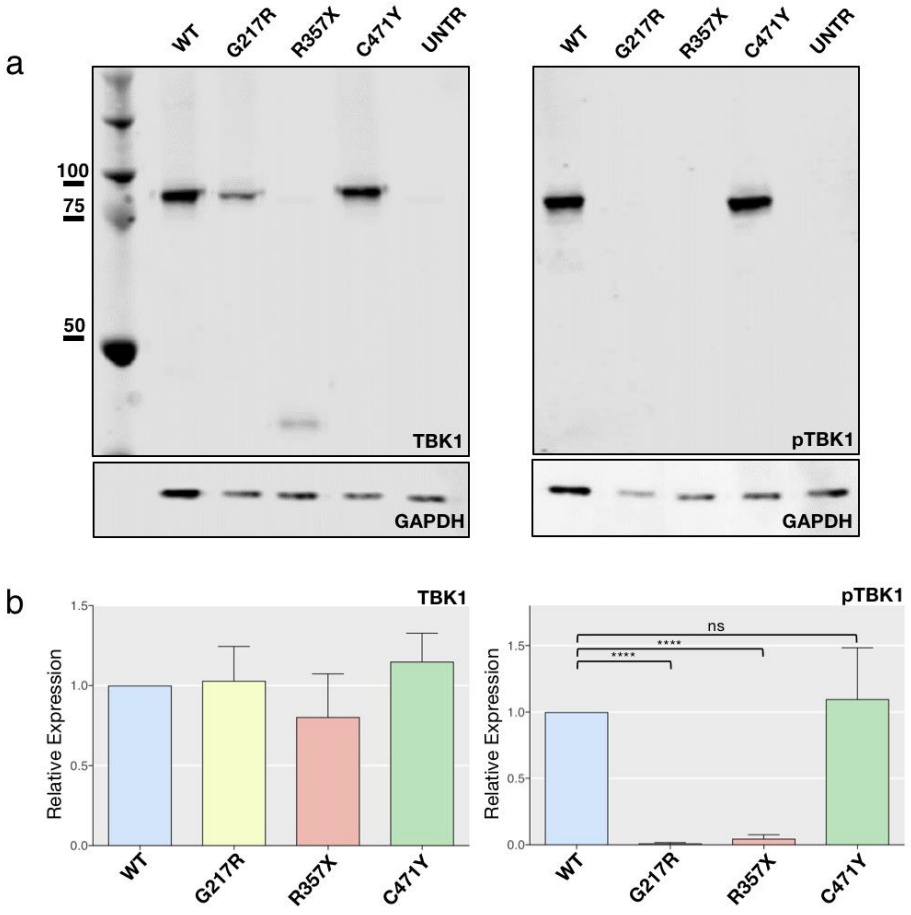


Figure 3.6 - TBK1 p.G217R and p.R357X impair TBK1 binding with OPTN and its phosphorylation. (a) Co-IP with HA tag pull down (TBK1) showing no binding of OPTN in any of the mutated samples with the exception of p.C471Y (n=3). (b) HEK293T were transiently co-transfected with Flag-OPTN WT and HA-TBK1 WT, p.G217R, p.R357X or p.C471Y, treated with alkaline phosphatase and analysed by western blot showing lack of OPTN phosphorylation in all mutated samples a part from p.C471Y.

3.4.6 ALS-linked *TBK1* variants decrease its phosphorylation

Since TBK1 phosphorylation is a fundamental aspect of the TBK1 activation, the variants p.G217R, p.R357X and p.C471Y were tested for TBK1 phosphorylation. Western blots of

HEK293T cells, transiently transfected with each variant, were probed with an antibody specific for phosphorylated Serine at position 172. Total TBK1 expression was similar among WT and all of the ALS-associated variants (Figure 3.7a,b). Robust levels of phospho-Ser172 TBK1 were evident in WT and p.C471Y transfected cells but were absent in cells expressing p.G217R and p.R357X variants (Figure 3.7a,b, $p<0.0001$). Transfected HEK293T cells were probed for phopsho-S172 TBK1 by immunocytochemistry, which confirmed that TBK1 phosphorylation was absent in cells expressing p.G217R and p.R357X mutants (Figure 3.7c). This indicates that the p.G217R and p.R357X but not the p.C471Y variant abolished the capacity of TBK1 for auto-phosphorylation as well as the ability of TBK1 to be phosphorylated.



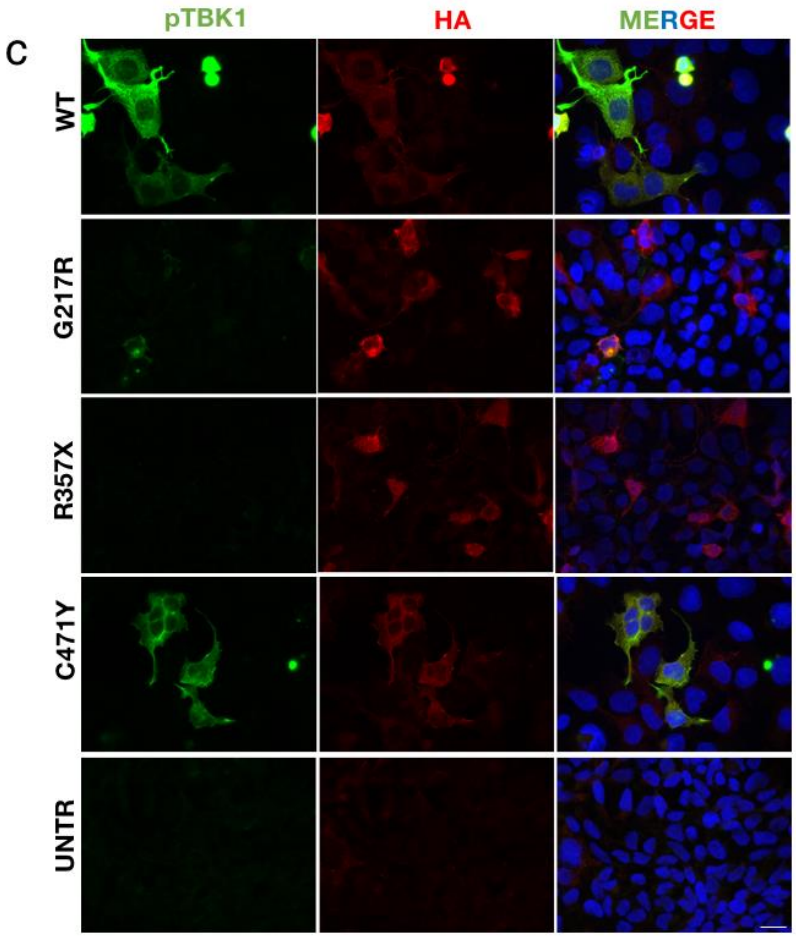


Figure 3.7 - TBK1 p.G217R and p.R357X impair TBK1 phosphorylation and auto phosphorylation. (a) Western blot analysis of TBK1 expression levels (left) and pTBK1 (right) (b left) Quantitative analysis of blot in (a) left showing a similar expression level of TBK1 in cells (n=4, analysed by One Way ANOVA followed by Dunnett's correction p<0.6754). (b right) Quantitative analysis of blot in (a) right showing a significant decrease of expression of pTBK1 in p.G217R and p.R357X (n=4 analysed by One Way ANOVA followed by Dunnett's correction p<0.0001). Error bars represent the standard error of the mean (SEM) (c) Qualitative immunocytochemistry of HEK293T transfected with TBK1-WT, p.G217R, p.R357X and probed for p-TBK1 confirming the result obtained by Western Blot analysis (Scale bar = 10 µm)

3.4.7 ALS-associated TBK1 variant p.G217R disrupts TBK1 homo-dimerisation

TBK1 has to homo-dimerise in order to be functional and does so via a central axis formed by aligning the two SDD domains in a parallel orientation. The ULD and KD domains interact with each other at one end of the dimer creating a globular structure and stabilising the homodimer (Tu et al., 2013) (Figure 3.4d, described in section 3.1.1). In order to investigate whether any of the variants analysed disrupted TBK1 homo-dimer

formation, HEK293T cells were transfected with TBK1 WT, p.G217R, p.R357X and p.C471Y and run on non-denaturing gels, which lack SDS that would unfold the protein and disrupt the hydrostatic bonds between dimers (Figure 3.8a). Native blots revealed two strong high and low molecular weight bands for WT and p.C471Y TBK1, indicating that a similar proportion exists as a homodimer and monomer. Little dimerisation was evident for the p.G217R mutant and no band was visible for the p.R357X truncation mutant (Figure 3.8a,b). When quantified, p.G217R showed a significantly lower ratio of TBK1 dimer/monomer compared to TBK1 WT (p=0.0306) (Figure 3.8b).

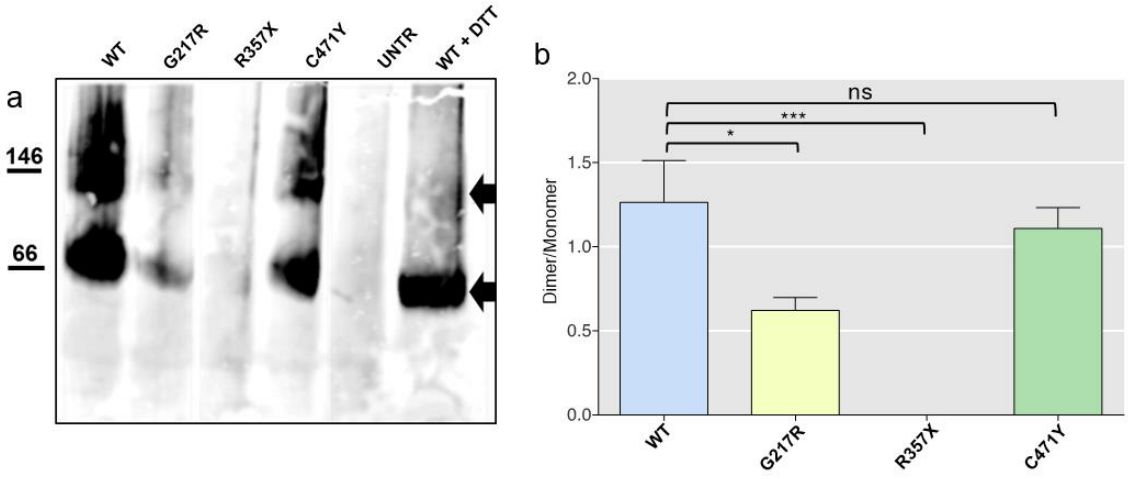


Figure 3.8 - TBK1 p.G217R reduces TBK1 homodimerisation. (a) Native gel showing dimer and monomer (indicated by black arrows) in TBK1-WT and TBK1-C471Y, weaker dimer and monomer in p.G217R sample and no dimer or monomer in R357X sample (b) quantitative analysis of gel in (a) showing significant reduction in dimer formation for p.G217R (positive control on the right TBK1 WT treated with DTT, n=3, analysed by One Way ANOVA followed by Dunnet's correction p=0.0009)

3.4.8 TBK1 variant c.992+1G<A produces an in-frame deletion of exon 8

The c.992+1G<A variant, also observed in ALSdb (Table 3.3), mutates the same base as a previously reported splice mutation (c.992+1G<T) that resulted in an in frame deletion of exon 8, within the ULD (Gijselinck *et al.*, 2015). More recently, another splicing mutation was reported (c.992+4_992+7delAGTA) in a FTD-ALS case that is predicted to produce the same in frame deletion (p.Gly272_Thr331del) (van der Zee *et al.*, 2017). In order to investigate if the variant c.992+1G<A had the same effect, cDNA

was made from a lymphoblastoid cell line (LCL) derived from the patient harbouring c.992+1G<A variant. cDNA from patient and control lines was amplified using primers enclosing the whole of the TBK1 gene. When PCR products were ran on an agarose gel we observed a deletion of ~ 200 bp corresponding to the size of exon 8 (Figure 3.9). The PCR product was then Sanger sequenced in house, which revealed the presence of a double sequence starting from the very first base of exon 8, confirming the result obtained with the agarose gel (Figure 3.9).

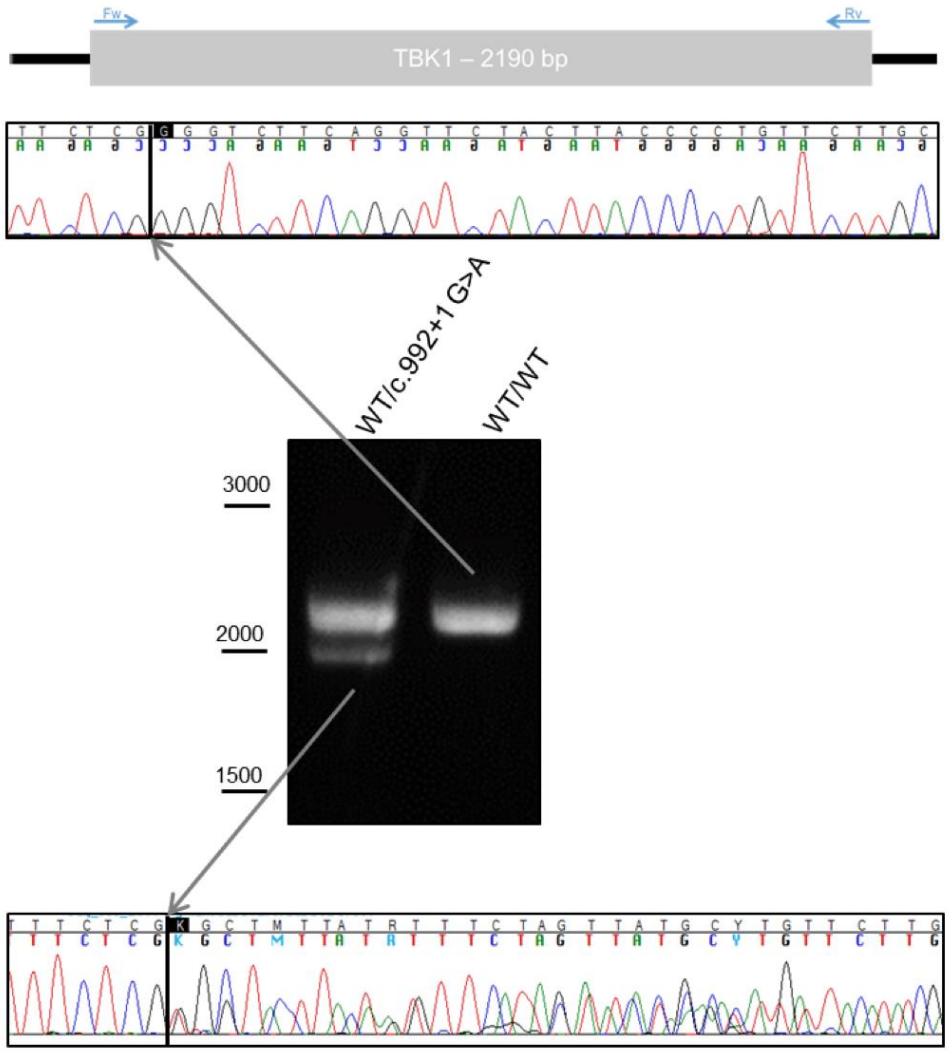


Figure 3.9 - TBK1 variant c.992+1G<A produces in frame deletion of exon 8. Agarose gel of cDNA extracted from WT and c.992+1G<A (p.G272_T331del) LCL showing two different sized bands for the WT allele and the mutated allele carrying c.992+1 G<A compared to the sample carrying both WT alleles (WT/C992+1G>A WT allele: 2190 bp, WT/C992+1G>A mutated allele: 1990 bp, WT/WT: 2190 bp). The

boxes above and below show the sequencing of the WT/WT sample and the sequencing of the WT/c.992+1G<A sample, respectively.

3.4.9 Characterisation of patient derived LCLs harbouring TBK1 variants

In order to assess whether *TBK1* variants decrease endogenous phospho-TBK1 levels, the amount of total TBK1 and phospho-TBK1 was measured in five patient derived LCLs heterozygous for *TBK1* variants: p.T31A, p.G272-T331del, p.R358H, p.Q565P, p.E643del compared to seven control derived LCLs. Western blot analysis revealed that total TBK1 levels in mutant and control LCLs were similar (p=0.6054, Figure 3.10a,b). Western blot analysis of phopsho-S172-TBK1 revealed a lower level of TBK1 phosphorylation in all mutant lines (p=0.0614 Figure 3.10a,b).

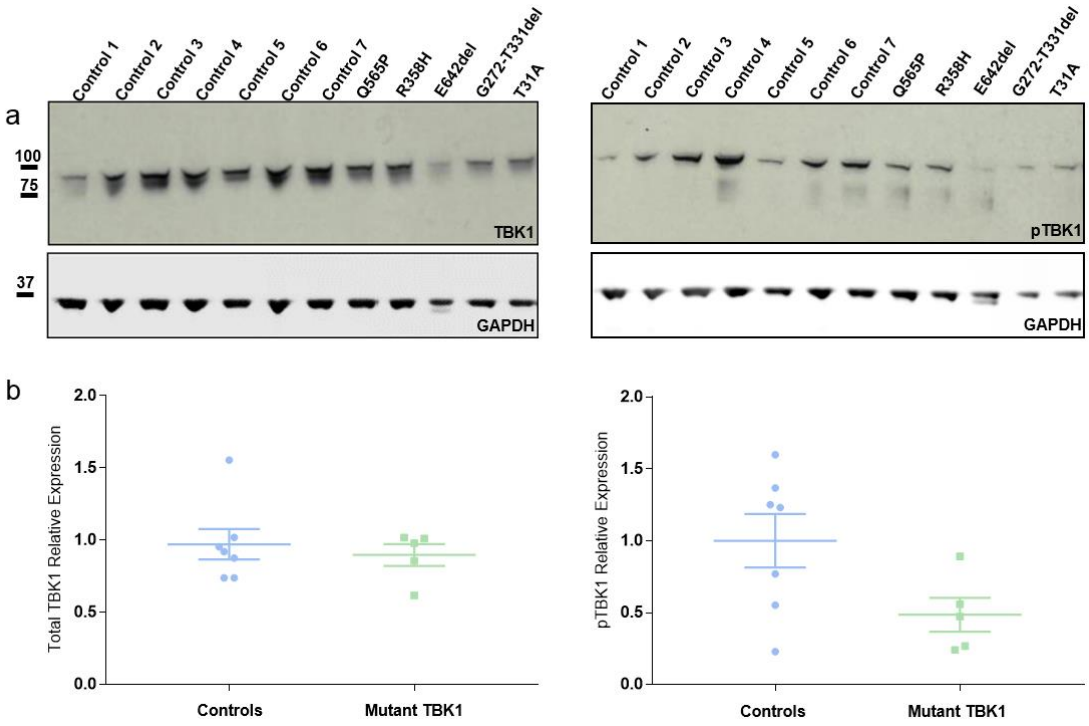


Figure 3.10 - Patient derived LCLs harbouring TBK1 variants show reduced level of TBK1 phosphorylation. (a left) Western blot analysis of TBK1 expression levels in patient and control derived LCLs. (a right) Western blot analysis of phospho-TBK1 expression levels in patient and control derived LCLs. (b) quantification of TBK1 (right, n=5) and phopho-TBK1 (left, n=3) showing similar levels of TBK1 mutant and control lines (analysed by unpaired t test, two tailed p=0.6054) and reduced levels of phopsho-TBK1 in all mutant samples (analysed by unpaired t test, two tailed p=0.0614). Error bars represent SEM.

When the level of phospho-TBK1 in the different lines was adjusted for the level of total TBK1 and controls were compare against mutant lines, an unpaired t test revealed a significant difference in the phopsho-TBK1/totalTBK1 between control and patient LCLs lines (p=0.0229, Figure 3.11).

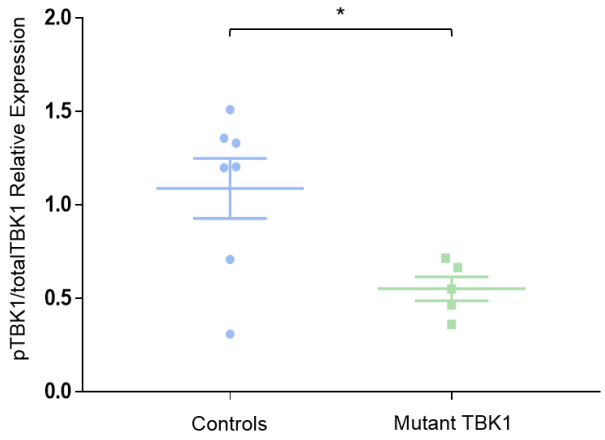


Figure 3.11 – pTBK1/totalTBK1 shows a significant difference between patient and control LCLs. Quantification of the ratio between phospho-TBK1 and total TBK1 (n=3) showing a significantly higher ratio in control compared to patient derived LCLs (analysed by unpaired t test, two tailed p=0.0229). Error bars represent SEM.

3.4.10 TOM20 and COXIV maintain similar expression in HEK293T cell transiently transfected with ALS-linked *TBK1* variants

As previously described, TBK1 has been shown to be involved in autophagy and more specifically in mitophagy (section 3.1.3.1). For this reason, expression of mitochondria markers was checked by western blotting in HEK293T cells transiently transfected with p.G217R, p.R357X and p.C471Y. No difference was detected in the expression of translocase of outer mitochondrial membrane 20 (TOM20), protein which is located in a mitochondrial receptor complex (TOM), or in the expression of cytochrome C oxidase subunit IV (COXIV), a marker for the mitochondrial electron transport chain (Figure 3.12).

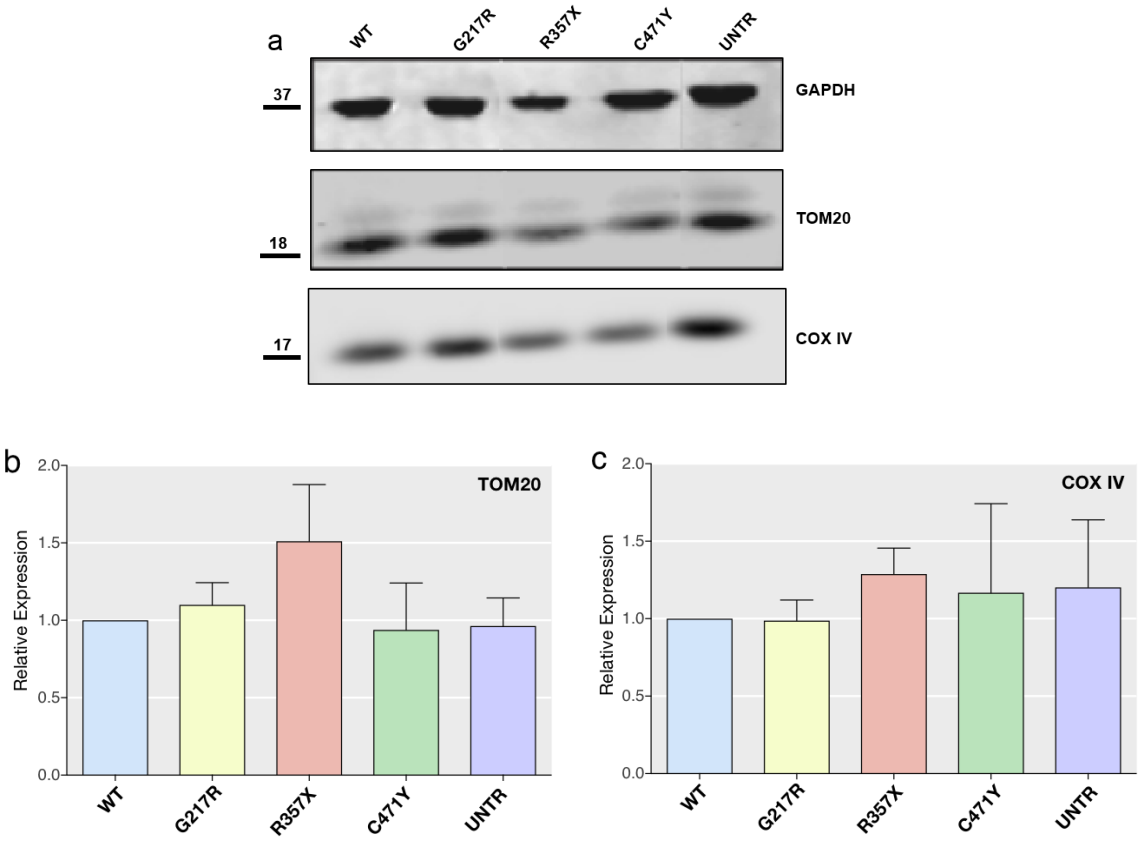


Figure 3.12 - TOM20 and COXIV maintain similar expression in HEK293T cell transiently transfected with ALS-linked TBK1 variants. (a) Western Blot analysis of TOM20 and COXIV expression levels in HEK293T cells transiently over-expressing HA-TBK1 WT, p.G217R, p.R357X or p.C471Y and untransfected cells (UNTR=untransfected) (b) Quantitative analysis of blot in (a) showing a similar expression level of TOM20 across samples (analysed by One Way ANOVA followed by Dunnett's correction p=0.4523). (c) Quantitative analysis of blot in (a) showing a similar expression level of COXIV across samples (analysed by One Way ANOVA followed by Dunnett's correction p=0.9580). Error bars represent SEM.

3.5 Discussion

This study shows the systematic analysis of samples from 699 index fALS patients that were devoid of mutations in known ALS genes. From this analysis an enrichment in variants was observed in *TBK1*, a gene recently associated with ALS (Cirulli et al. 2015; Freischmidt et al. 2015). The missense variants predominantly map within the functional domains of TBK1, and were most frequent in the kinase and ubiquitin-like domains (Figure 3.4a,b). Interestingly, clustering of missense and nonsense variants was evident

in the ULD domain (Figure 3.4a,b), which has been reported to be important in TBK1 homo-dimerisation (Li *et al.*, 2012; Tu *et al.*, 2013).

We identified 16 *TBK1* variants, of which four were nonsense, predicted to generate mRNA degraded by nonsense mediated decay or to be translated as truncated proteins, three were in frame deletions and nine missense. Four of these variants (p.M623fs, p.Q629fs, T31A, R358H) are novel. Twelve of these variants have previously been reported (p.Q2X, p.T79del, p.R357X, p.E643del, p.N22H, p. N129D, c.992+1A<G/p.G272_T331del, p.G217R, p.R357Q, p.Y394D, p.C471Y and p.Q565P). Of these only p.R357Q, p.E643del and T79del have been functionally investigated (Freischmidt *et al.*, 2015; Gijselinck *et al.*, 2015; van der Zee *et al.*, 2017). Mutations at the same nucleotide c.992+1G<T (p.G272_T331del) and c.992+4_992+7delAGTA (p.G272_T331del) have previously been reported to splice out the whole of exon 8 (Gijselinck *et al.*, 2015; van der Zee *et al.*, 2017), resulting in an in frame partial deletion of the ULD, a finding that was replicated for c.992+1G<A (Figure 3.9). Furthermore, the patient harbouring the variant p.R358H was found to carry the *FUS* mutation p.R521C. Interestingly, Freischmidt and colleagues also reported the presence of a *TBK1* variant (p.Y185X) in a patient harbouring the p.R524G *FUS* mutation (Freischmidt *et al.*, 2015). This, together with the observation that the patient harbouring TBK1 variant p.Y394D was found to carry the p.M337V *TARDP* mutation, supports the theory of an oligogenic basis of ALS, postulated by others (Van Blitterswijk, Van Es, *et al.*, 2012). Interestingly, the patient harbouring *TBK1* p.Y394D and *TARDP* p.M337V displays the earliest age of onset in the cohort here presented (Table 3.3), which is abundantly below the average age of onset described in literature (Table 3.1). This phenomenon was also described by Freischmidt and colleagues in the patients (all three affected relatives) harbouring *TBK1* p.Y185X and *FUS* p.R524G (Freischmidt *et al.*, 2015).

Functional investigation was carried out on three variants selected on the basis of their predicted disruption to TBK1 structure and homo-dimerisation (Figure 3.4d). The variants chosen were: p.G217R as it lies within the kinase domain (KD) and was found in a

putative affected sibling; p.R357X as it lies within the ubiquitin like domain (ULD) and was found in an unrelated case in ALSdb; and p.C471Y, which lies within the scaffold dimerization domain (SDD). Only p.G217R and p.R357X TBK1 variants, but not p.C471Y, abolished the phosphorylation of IRF3 (Figure 3.5). This has previously been described for other ALS associated variants (Freischmidt *et al.*, 2015; Kim *et al.*, 2016; Tsai *et al.*, 2016). The same two TBK1 variants, p.G217R and p.R357X, also abolished TBK1 binding to OPTN and prevented its phosphorylation (Figure 3.6). The inhibition of TBK1 binding to OPTN has been previously observed in ALS *TBK1* variants, mainly located in the C-terminal region of the protein (p.L59Ffs, p.R444X, p.Ile472Serfs*8, p.M559R, p.E696K, p.690-713del) (Freischmidt *et al.*, 2015; Kim *et al.*, 2016; Tsai *et al.*, 2016; Pozzi *et al.*, 2017). Phosphorylation is necessary for OPTN to be activated and function as an autophagy receptor (Richter, et al. 2016). The impairment of OPTN phosphorylation by TBK1 suggests a link between ALS associated *TBK1* variants and the autophagy pathway. However, no difference in the expression of mitochondria markers, TOM20 and COXIV, was observed among HEK293T cells transfected with mutant TBK1 constructs, at least in normal conditions (Figure 3.12), which would suggest no direct involvement of these variants in the mitophagy pathway. However, this might change should the cells be put in a stressful environment following an exogenous challenge such as heat shock treatment, oxidative or endoplasmic reticulum stress. Furthermore, it has previously been shown that OPTN and TBK1 are recruited in proximity of damaged mitochondria to direct their autophagic digestion (Richter et al. 2016). Looking at TBK1, OPTN and mitochondria co-localisation in HEK293T cells transiently transfected with the TBK1 variants analysed in this study, may well shed light on their involvement in the mitophagy pathway.

To become activated TBK1 must form a homo-dimer and phosphorylate itself or be phosphorylated by other kinases (described in section 3.1.1). Here we show that the same two *TBK1* variants found to impair OPTN binding as well as OPTN and IRF3 phosphorylation, affect TBK1 phosphorylation itself (including auto-phosphorylation,

Figure 3.7). This observation is in line with a recent study that showed the lack of TBK1 phosphorylation in ALS-associated *TBK1* in frame deletions (p.T79del, p.D167del, p.E643del) (van der Zee *et al.*, 2017). We also show that p.G217R affects TBK1 ability to homodimerise, although located the KD. However, a limitation of this study is having used a non-denaturing gel that shows high background noise and is difficult to quantify accurately. Furthermore, no band was detected for p.R357X in any of the three replicates. A confirmation of this result via co-IP with TBK1 WT bearing a different tag (i.e. GFP or FLAG), would give further support to this conclusion.

TBK1 phosphorylation and dimerisation are two pivotal properties for its function. In contrast to the result from all our functional studies, the p.C471Y variant, which lies in the SDD, is able to phosphorylate and homo-dimerise at similar levels to wildtype TBK1 protein and therefore if it does play a pathogenic role, it is likely to be due to a different mechanism.

Lastly, the ALS patient derived lymphoblastoid lines analysed in this study harbouring TBK1 mutations, show reduced TBK1 phopshorylation, approaching significance (Figure 3.10). When corrected for the amount of total TBK1, the difference in phosphorylation between patient and controls became significant (Figure 3.11), suggesting that there is indeed a lower level of TBK1 phosphorylation of LCLs derived from patients harbouring the five mutations p.T31A, p.G272-T331del, p.R358H, p.Q565P, p.E643del, when compared to controls. This supports the hypothesis that disease linked TBK1 variants might impair TBK1 phosphorylation, disrupting its ability to bind and phosphorylate multiple partners including OPTN, an important component in the autophagy pathway (OPTN's role in autophagy will be further discussed in section 4.1.2). We also found that total TBK1 was expressed in similar amount in all the lines. This result supplies further evidence that haploinsufficiency is a possible disease mechanism for TBK1.

3.6 Conclusions

In conclusion, this study identified four novel and twelve previously described ALS-associated *TBK1* variants. Functional studies on three of those (p.G217R, p.R357X and p.C471Y) suggest that nonsense mutations in the kinase domain (p.G217R) and truncation mutation in the ubiquitin-like domain (p.R357X) impair TBK1 kinase activity and its binding to OPTN. We also show that p.G217R significantly reduces TBK1 ability to homodimerise when compared to wild type. This, in turn, prevents auto-phosphorylation or phosphorylation of TBK1 by other kinases into its active form as well as the phosphorylation of other TBK1 substrates such as IRF3 and OPTN. Thus, missense mutations in critical functional domains may also cause disease through a loss of TBK1 function similar to the truncation mutations. Our results in overexpression models and endogenously expressed TBK1 variants, further support the hypothesis that TBK1 mutations result in haploinsufficency as a possible mechanism of pathogenesis (Cirulli et al. 2015; Freischmidt et al. 2015, 2016). Further investigation of how ALS-linked *TBK1* variants alter TBK1 structure, phosphorylation and dimerisation will help unravel the disease pathogenesis and identify novel therapeutic targets.

Chapter 4 – A novel *Optineurin* truncation mutation identified in an adult onset consanguineous Palestinian family with ALS

4.1 Background

Optineurin (*OPTN*) mutations have been associated with a number of disorders including normal tension glaucoma (NTG) (Rezaie *et al.*, 2002), Paget's disease of the bone (Albagha *et al.*, 2010; Obaid *et al.*, 2015), Crohn's disease (Smith *et al.*, 2015) and ALS (Maruyama *et al.*, 2010). *OPTN* encodes for a highly conserved 577 amino acid protein (OPTN) involved in many important cellular pathways such as cell division, autophagy, vesicle trafficking and defence against pathogens (Ying and Yue, 2016; Markovinovic *et al.*, 2017). OPTN protein inclusions have been shown to colocalise with protein aggregates in many neurodegenerative diseases not associated with mutations in OPTN, including Alzheimer's, Parkinson's and Huntington's disease, which provides further evidence that OPTN is involved in proteostasis (Maruyama *et al.*, 2010; Hortobágyi *et al.*, 2011).

4.1.1 Structural insight of OPTN

Located on chromosome 10, *OPTN* contains 16 exons of which 13 are coding. OPTN is a ubiquitously expressed protein, with highest presence in brain, retinal ganglion cells, heart, spleen and skeletal muscle (Li, Kang and Horwitz, 1998; Rezaie *et al.*, 2002, 2005; De Marco *et al.*, 2006; Ying and Yue, 2012). OPTN has five functional domains: two coiled coil domains, CC1 (aa 38-170) and CC2 (aa 238-509), an LC3 interacting region (LIR, aa178-181), a ubiquitin binding region of A-20 binding inhibitors of NF-kB (ABIN) proteins and NF-kB modulators (NEMO) (UBAN, aa 461-493) and a zinc finger domain (ZF, aa 553-557) with unclear function (Zhu et al., 2007; Morton et al., 2008; Laplantine et al., 2009; Wild et al., 2011; Liu et al., 2014; Li et al., 2016) (Figure 4.1).

4.1.1.1 Coiled coil domains

CC1 and CC2 are hydrophobic sequences that cover 70% of OPTN surface (Markovinovic *et al.*, 2017). They are known to trigger interaction with hydrophobic regions such as other CC domains (CCDs). Therefore, it has been proposed that OPTN forms oligomers with itself through its CCDs (Ying *et al.*, 2010; Gao *et al.*, 2014). These interactions can be cross-linked in case of oxidative stress (Gao *et al.*, 2014). OPTN also uses its CCDs to interact with other proteins such as TBK1 (Li *et al.*, 2016), which is discussed further in section 3.1.3.

4.1.1.2 LC3 interacting region

The LIR domain is a common feature of autophagy receptors as it allows OPTN and other autophagy receptors such as p62 and NDP52, to recognise LC3 and deliver its cargo to the autophagosome (Wild *et al.*, 2011). OPTN's ability to function as an autophagy receptor relies heavily on its activation by phopsho-kinases, particularly by TBK1 (Pilli *et al.*, 2012).

4.1.1.3 Ubiquitin-binding region of ABIN proteins and NEMO (UBAN)

The UBAN domain in OPTN is mainly devoted to ubiquitin chain recognition, enabling it to bind polyubiquitinated proteins and recruit them to the autophagosome through its LIR domain binding to LC3 (Wild *et al.*, 2011).

4.1.2 OPTN in autophagy

OPTN is involved in different forms of autophagy, which can be ubiquitin-dependent or ubiquitin-independent as described below.

4.1.2.1 OPTN in Xenophagy

Xenophagy is a specific subtype of autophagy that selectively recognises the ubiquitinated pathogens and brings them to the autophagophore (Randow and Youle,

2014). The role of OPTN, as well as TBK1, in this type of autophagy has been extensively studied in the clearance of *Salmonella* bacteria as OPTN's affinity to LC3 is greatly enhanced by the phosphorylation of its Ser177 by TBK1 (Wild *et al.*, 2011).

4.1.2.2 Mitophagy

The role of OPTN in miotophagy involves the delivery of polyubiquitin tagged malfunctioning mitochondria to the autophagophore (illustrated in section 1.6.2; Wong and Holzbaur 2014). The loss of OPTN inhibits LC3 mediated recruitment and degradation of defective mitochondria, which can be reversed by the expression of WT OPTN but not of ALS-linked mutant p.E478G OPTN, which impairs the function of the UBAN domain. The same outcome was observed when expressing OPTN constructs with mutations in the LIR domain (Wong and Holzbaur, 2014).

4.1.2.3 Aggrephagy

Aggrephagy is a form of autophagy that selectively targets large protein aggregates. OPTN has been shown to play a role in the degradation of mutant hungtintin (mHtt) in an ubiquitin-dependent manner (Shen *et al.*, 2015). OPTN can also be involved in aggrephagy in an ubiquitin-independent manner as it was found to co-localise with SOD1 p.G93C and Htt aggregates even when carrying the p.E478G mutation, which disrupts OPTN binding with ubiquitin. This suggests that OPTN can still be involved in the degradation of these aggregates even if devoid of its ability to bind ubiquitin (Korac *et al.*, 2013).

4.1.3 OPTN in vesicle and membrane trafficking

OPTN has been shown to interact with a number of proteins involved in intracellular vesicle trafficking such as myosin VI and Rab8. Myosin VI is a multifunctional motor protein that binds to OPTN through its C-terminal region. When OPTN is knocked down, myosin VI is no longer recruited to the Golgi apparatus and the process of exocytosis is dramatically reduced (Sahlender *et al.*, 2005). The binding of OPTN to myosin VI

induces its dimerization, which triggers the movement of myosin VI towards the minus end of the actin filaments (Phichith *et al.*, 2009). Since OPTN also functions as an autophagy receptor, it binds the autophagosome through its LIR and carries the whole complex inducing their fusion with the lysosome (Tumbarello *et al.*, 2012). Indeed, knock down of OPTN in HeLa cells inhibits autophagy (Wild *et al.*, 2011) and exocytosis (Sahlender *et al.*, 2005). Decreased interaction between OPTN and myosin VI has been reported in the spinal cord tissue of apparently sporadic ALS patients, suggesting that disruption of OPTN function could impair the autophagy pathway in ALS (Sundaramoorthy *et al.*, 2015).

4.1.4 OPTN in neuroinflammation

Toll like receptors (TLRs) and cytosolic DNA and RNA receptors activate two mechanisms: pro-inflammatory cytokine synthesis, regulated by NF-kB (Sen and Baltimore, 1986; Medzhitov, Preston-Hurlburt and Janeway, 1997) and the TBK1 pathway, which regulates the transcription of IFN type I production (Akira, Uematsu and Takeuchi, 2006).

Neuroinflammation has been associated with ALS in mutant SOD1 mouse models, where microglial activation was found to precede motor neuron symptoms (Hall, Oostveen and Gurney, 1998). This result was confirmed in patients whose PET scans reported extensive microglial activation (Turner *et al.*, 2004). Interestingly, OPTN has been found to suppress TNF-mediated NF-kB activation when overexpressed in HEK293 cells (Zhu *et al.*, 2007; Nagabhushana, Bansal and Swarup, 2011). In support of this data, ALS-associated OPTN variants lack the ability to suppress NF-kB in neuroblastoma spinal cord 34 (NSC-34) lines (Zhu *et al.*, 2007; Maruyama *et al.*, 2010; Akizuki *et al.*, 2013; Nakazawa *et al.*, 2016). However, recent studies have shown that immune cells derived from OPTN deficient mice may activate NF-kB equally well (Gleason *et al.*, 2011; Munitic *et al.*, 2013; Slowicka *et al.*, 2016). Therefore, this topic remains controversial. Markovinovic and colleagues have recently published a review

that suggests that OPTN carries out a neuroprotective role by inhibiting inflammation and necroptosis, a caspase-independent subtype of cell death that resembles necrosis (Markovinovic *et al.*, 2017).

4.1.5 OPTN in ALS

OPTN was linked to ALS in 2010 when a Japanese study found two nonsense and one missense mutations in OPTN by homozygosity mapping in four consanguineous families affected by ALS (Maruyama *et al.*, 2010). Around 40 *OPTN* variants have been found in ALS patients since then (Figure 4.1) with a great variability in terms of frequency and penetrance, which may depend on the patient's ethnicity. Mutations in *OPTN* appear to occur with higher frequency in Chinese and Japanese populations (3% in fALS and 1% in sALS against a total frequency of 1% in Caucasian populations) (Maruyama *et al.*, 2010; Del Bo *et al.*, 2011; Iida *et al.*, 2012; Tümer *et al.*, 2012; Van Blitterswijk, Van Vught, *et al.*, 2012; Weishaupt *et al.*, 2013; Soong *et al.*, 2014; Li *et al.*, 2015; Özoğuz *et al.*, 2015; Fifita *et al.*, 2016). To date, only a few *OPTN* mutations have been published in non-Asian populations, suggesting a genetic founder effect (Markovinovic *et al.*, 2017). In support of this, the homozygous variant c.691_692insAG (p.S121fs*) was recently found to recur at a frequency of 5.8% and 0.3% in a Moroccan and an Ashkenazi Jewish population respectively (Goldstein *et al.*, 2016).

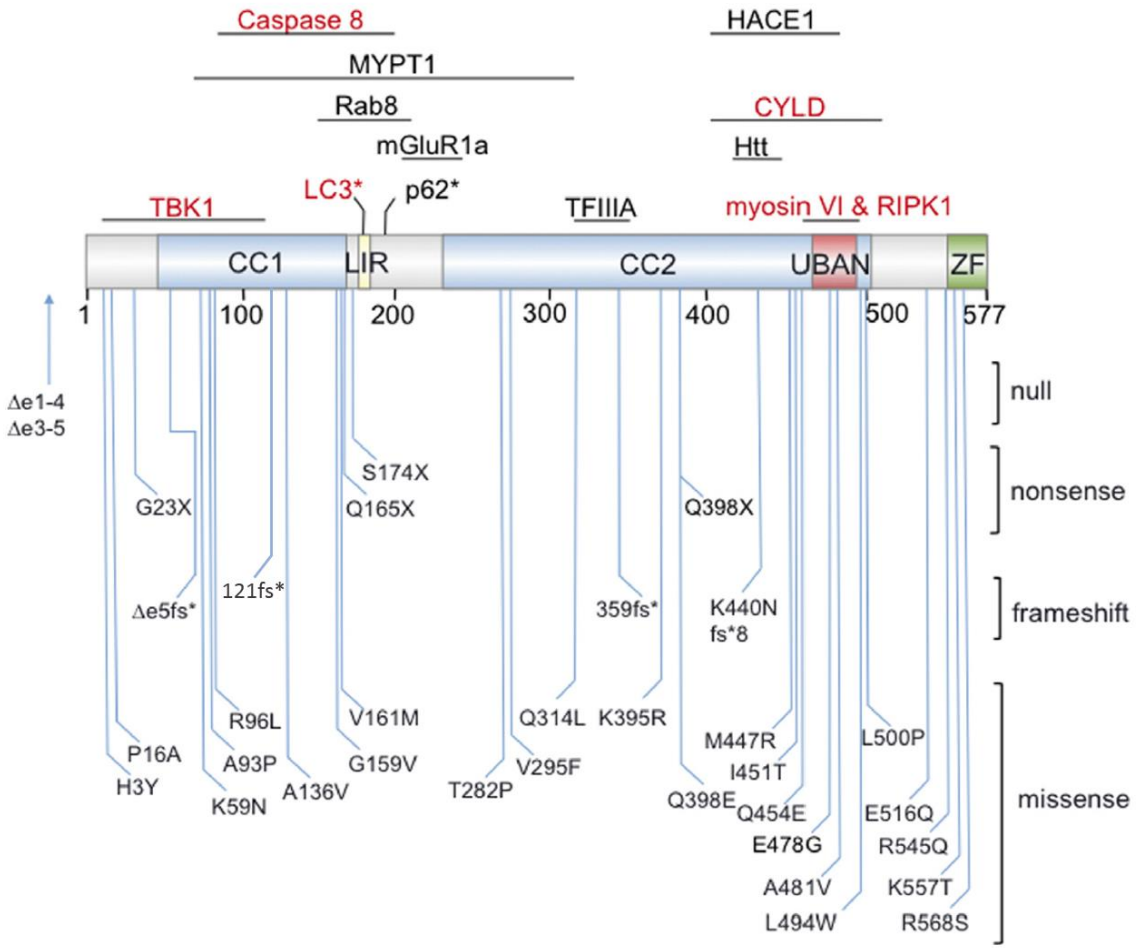


Figure 4.1 – OPTN structure, binding partners and ALS-associated variants found to date (adapted from Markovinovic et al. 2017)**.** The figure shows the five OPTN domains (Zhu *et al.*, 2007; Morton *et al.*, 2008; Laplantine *et al.*, 2009; Gleason *et al.*, 2011; Liu *et al.*, 2014; Li *et al.*, 2016), OPTN binding partners and their binding regions (* indicates the need of S177 to be phosphorylated in order for LC3 to bind and K193 to be polyubiquitinated for p62 to bind). The diagram also shows all the *OPTN* variants found in ALS patients to date divided by null nonsense, missense. Δe = exon deletion. Above are OPTN binding partners and their regions of interaction. In red are the proteins that are believed to be involved in neuroprotection (MYPT1= Myosin phosphatase target subunit 1; mGLuR1a= Metabotropic Glutamate Receptor 1A; TFIIIA= Transcription Factor for polymerase III A; CYLD=Ubiquitin carboxyl-terminal hydrolase; HACE= HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1; RIPK1= Receptor Interacting Serine/Threonine Kinase 1).

Clinical reports suggest that OPTN mutations cause a moderately progressive phenotype with long progression prior to respiratory failure (Maruyama *et al.*, 2010) and occasionally features of aphasia and FTD (Pottier *et al.*, 2015; Minegishi *et al.*, 2016).

The more frequently hypothesised mechanism for OPTN pathogenicity is its loss of function, including haploinsufficiency, by null and nonsense mutations. It is noteworthy that most *OPTN* nonsense mutations have been found as homozygous variants appearing to cause disease in an autosomal recessive fashion. However, the same nonsense homozygous mutations (excluding p.Q398X), as well as many deletion and missense mutations, have also been found as heterozygous variants, suggesting that haploinsufficiency might be enough to induce ALS (Tümer *et al.*, 2012; Goldstein *et al.*, 2016).

Around 20 potentially damaging missense *OPTN* variants have been found in ALS patients (Maruyama *et al.*, 2010; Del Bo *et al.*, 2011; Millecamps *et al.*, 2011; A. Chiò *et al.*, 2012; Naruse *et al.*, 2012; Van Blitterswijk, Van Vught, *et al.*, 2012; Lattante *et al.*, 2012; Weishaupt *et al.*, 2013; Kenna *et al.*, 2013; Soong *et al.*, 2014; Beeldman *et al.*, 2015; Morgan *et al.*, 2015; Özoğuz *et al.*, 2015; Li *et al.*, 2015; Bury *et al.*, 2016; Fifita *et al.*, 2016), however, their mechanism of pathogenicity is largely unknown. A clustering of these variants is observed at the C-terminal region of OPTN, responsible for its oligomerisation and binding with ubiquitin (Figure 4.1). The p.E478G OPTN mutation, associated with ALS in 2010, has been the most extensively studied. This variant has been shown to:

- inhibit binding with ubiquitin (Mao *et al.*, 2017) and myosin VI (Sundaramoorthy *et al.*, 2015),
- fail to suppress the NF-kB pathway (Maruyama *et al.*, 2010; Nakazawa *et al.*, 2016),
- to cause demyelination of corticospinal tracts in post mortem tissues (Maruyama *et al.*, 2010),
- decrease OPTN solubility in human patient spinal cord lysate (Sundaramoorthy *et al.*, 2015).

These results support a loss of function mechanism, however, toxic gain of function cannot be completely excluded for this specific variant (Markovinovic *et al.*, 2017).

Interestingly, a recent study reported a patient with pathological features of FTD harbouring an *OPTN* (p.G538Efs*27) and a *TBK1* variant (p.R117X) that had no evidence of motor neuron degeneration. Analysis of post mortem tissue revealed atrophy of the frontotemporal lobe along with TDP-43 and p62 proteinopathy. Protein and mRNA levels of OPTN and TBK1 were decreased, supporting the hypothesis of haploinsufficiency as disease mechanism (Pottier *et al.*, 2015). Several reports describe detection of *OPTN* variants coinciding with *TARDP* or *C9ORF72* mutations, providing evidence that heterozygous *OPTN* mutations may be acting to increase disease risk in concert with mutations in other genes, supporting an oligogenic basis for disease pathogenesis in some cases of ALS and FTD (Weishaupt *et al.*, 2013; Pottier *et al.*, 2015).

4.1.6 OPTN in primary open angle glaucoma

ALS and primary open angle glaucoma (POAG) are two neurodegenerative diseases that affect different subtypes of neurons. Three missense and one nonsense *OPTN* variants were first associated in 2002 with normal tension glaucoma (NTG), a form of POAG (Rezaie *et al.*, 2002). The most widely studied *OPTN* variant found in NTG is p.E50K, which has been shown to cause disease by toxic gain of function (Markey *et al.*, 2015). Interestingly, this OPTN mutant shows an increased ability to bind TBK1 (Morton *et al.*, 2008; Li *et al.*, 2016). TBK1 is also thought to play distinct roles in ALS and NTG, whereby mutants can cause the disease through haploinsufficiency and toxic gain of function respectively.

4.1.7 OPTN mouse models

Mice knocked out for OPTN (optn-/-) display axonal loss and dysmyelination in the spinal cord, the same phenotype also observed when OPTN was selectively knocked out from

olygodendrocites, myeloid cells or microglia but not when knocked out from motor neurons or astrocytes (Ito *et al.*, 2016). Optn-/- mice also induced higher susceptibility of mice to *Salmonella* infections (Slowicka *et al.*, 2016). An $OPTN^{D477N/D477N}$ knock-in mouse model was also generated. The p.D477N OPTN mutant lacks the ability to bind to ubiquitin, however these mice did not show any motor neuron degeneration even after 12 months (Gleason *et al.*, 2011). In contrast, $OPTN^{I407X/I407X}$ show embryonic lethality with incomplete penetrance and the surviving animals express a truncated form of OPTN at low levels, however this model also shows no symptoms of motor neuron degeneration (Munitic *et al.*, 2013).

4.2 Aim of this project

The aim of this project was to genetically and functionally characterise a novel recessive OPTN variant (p.S174X) identified in a consanguineous Palestinian family with an aggressive adult onset ALS using primary fibroblast lines derived family members.

4.3 Material and methods

The skin biopsy and initial fibroblast culture was performed by collaborators in Israel, as described in Chapter 2. Techniques specific to this chapter are explained below.

4.3.1 Clinical data and sample collection

The family were seen by our collaborator, Neurologist Dr Marc Gotkine in an outpatient clinic at Hadassah Hospital. Because the affected individuals were siblings and the parents were known to be first cousins we predicted that the disease allele was recessive and could be identified by shared regions of homozygosity.

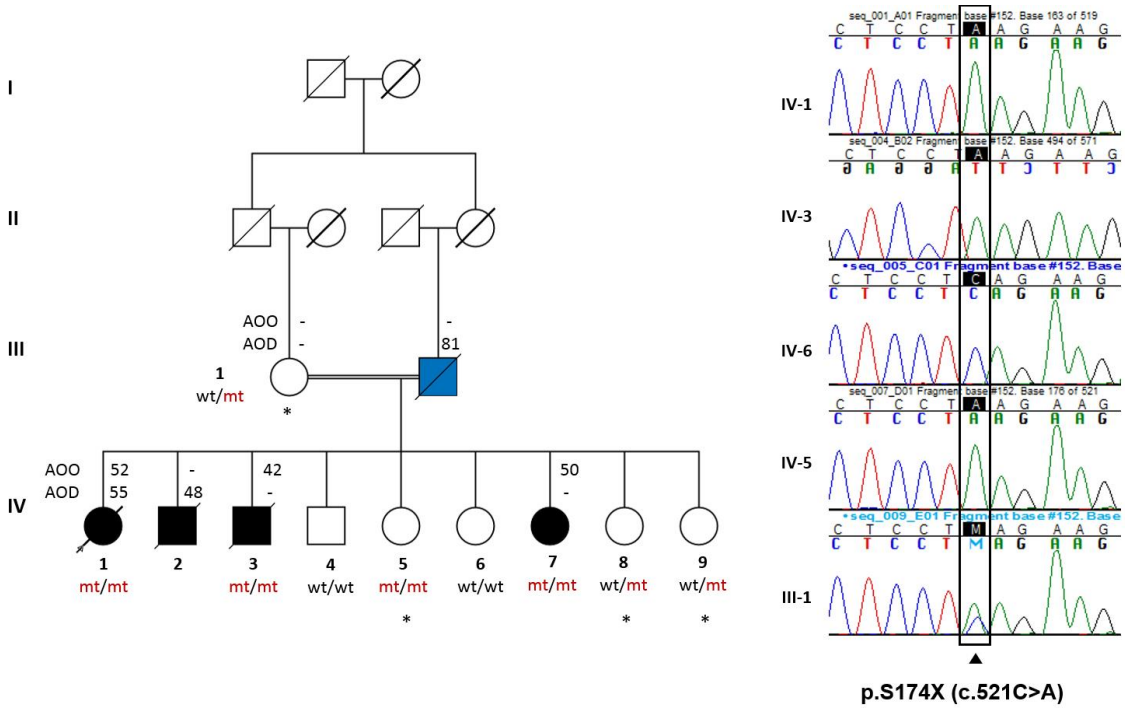


Figure 4.2 – Consanguineous family harbouring the p.S174X variant and sequencing confirming the phenotype of some of the components of the family. On the left, the consanguineous kindred carrying the *OPTN* variant p.S174X with genotype specified under every individual (when known). Female and male individuals are marked with round and square shapes respectively. The patients affected by ALS are marked with dark circle/square. The patient affected by Alzheimer is marked by blue square. Age of onset (AOO) and age of death (AOD) specified when known. The arrow indicates the proband and the stars indicate the individuals we obtained fibroblast lines from. On the left, the confirmation of the genotype by Sanger sequencing of five individuals belonging to the family: three homozygous for the p.S174X mutation (IV-1, IV-3, IV-5), one heterozygous (III-1) and one wild type (IV-6). Blue peaks indicate cytosine, red peaks indicate thymine, green peaks indicate adenine and black peaks indicate guanine. Sequencing was carried out by my fellow PhD student Chun Hao Wong.

DNA was obtained from the proband (IV-1), one affected brother (IV-3), one unaffected sister (IV-6) and the unaffected mother (III-1) (Figure 4.2). Skin punch biopsy explant culture from III-1, IV-5, IV-8 and IV-9 (Figure 4.2) individuals was carried out in Shaare Zedek Medical Centre as described in section 2.5.4. The pedigree in Figure 4.2 was drawn using Genial Pedigree Draw (www.pedigreedraw.com).

4.3.2 Shared regions of homozygosity

Following informed consent, blood samples were taken, DNA extracted and SNP genotyping was performed on individuals IV-1 and IV-3 using HumanOmniExpress-12v1-1 chips, which assess ~2.5 million bi-allelic variants distributed throughout the genome. Regions of shared homozygosity (RoH) were identified in Plink. In parallel their DNA was exome sequenced as described in section 2.2 and correlated with the SNP genotyping data by Simon Topp. 19,355 high quality homozygous variants were found and were filtered by the following criteria: protein changing variants, not present as a homozygote in any of the control databases analysed (670 KCL exomes; EVS/ESP; ExAC; GME exomes) and ExAC coverage by >10 reads in >50% of samples (Table 4.1). This analysis was carried out by my bioinformatician colleague Simon Topp.

4.4 Results

4.4.1 Clinical assessment of proband confirmed the diagnosis of ALS

The clinical assessment of these patients was carried out by a Neurologist, Dr Marc Gotkine. The proband's parents were first cousins. The father died at age 81 after being diagnosed with Alzheimer's disease three years prior to his death. The mother was 79 and healthy and the family denied the presence of neurological disease in previous generations. Three of their offspring developed neurological symptoms and were diagnosed with ALS: the proband (IV-1) and 2 of her brothers (IV-2 and IV-3) (Figure 4.2).

The proband presented at the age of 54 with a two year history of progressive gait difficulty, which she attributed to bilateral lower limb weakness. Roughly one year later she noted bilateral upper limb weakness followed within a few months by breathlessness, slurred speech and a dropped head. There were no visual, sensory or cognitive complaints and she denied sphincter involvement. Examination revealed tachypnea and use of accessory respiratory muscles and respiratory distress on lying

down. There were signs of upper and lower motor neuron involvement in all limbs and the cranial nerves. Elecromyography (EMG) confirmed the presence of LMN involvement in upper and lower limbs. Respiratory muscle weakness was confirmed by spirometry. Her condition continued to deteriorate, she declined assisted ventilation and died six months following diagnosis.

The family reported that one brother (IV-2) had recently died after suffering a similar illness, and a second brother (IV-3) had just been diagnosed as having a neuromuscular condition, which was later confirmed to be ALS. Although there was no indication of visual impairment in the proband, the wife of IV-2 reported that in the months before his death he became blind, however no ophthalmological exam was performed. Furthermore, another sibling (IV-7) has also recently reported similar motor symptoms.

4.4.2 Exome sequencing of two affected individuals reveals a novel *OPTN* mutation

Studying consanguineous families, or isolated populations, has been proven to be an effective methodology to discover novel ALS-linked variants (Yang *et al.*, 2001; Laaksovirta *et al.*, 2010; Maruyama *et al.*, 2010; Smith *et al.*, 2013). As described above, the Japanese study that identified the first ALS-linked mutations in OPTN has been an important advance in gene discovery, as the mutations found in their population have also been identified in populations of different ethnicity (Maruyama *et al.*, 2010).

Through a collaboration with Dr Marc Gotkine at Hadassah University Hospital in Israel, we were able to obtain DNA and perform exome sequencing on two members (IV-1 and IV-3, Figure 4.2) of this consanguineous family presenting an adult onset fast progressive form of ALS. Exome capture and exome sequencing were outsourced at the Centre for Genomic Research in Liverpool and at the Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust, King's College London, respectively. The rationale behind this approach is that consanguineous families have a higher risk of inheriting a homozygous variant (Hamamy, 2012), making the process of variant

discovery more efficient and cost effective in these families. Around 95% of the protein coding region of the genome were captured and sequenced to a depth higher than 10 reads in both patients, making it unlikely that a shared pathogenic variant will have been excluded from this analysis. The data obtained was further analysed by Simon Topp through a rigorous filtering strategy using public and private databases, illustrated in Table 4.1.

Filtering rationale	Number of variant
Total Unfiltered variants	1,569,097
Within probes and overhang	50,456
Homozygous	19,335
Protein changing	4,822
Not present in KCL controls as homozygous variant	100
Not present in EVS as homozygous variant	62
Not present in ExAC as a homozygous variant	27
Exac Coverage >50%	15
Shared in the two affected	2

Table 4.1 – Filtering strategy used to analyse the exome sequencing data from IV-1 and IV-3.

Two homozygous variants were identified as being novel and present in both individuals. The first one was p.A94T in *Lipase E* (*LIPE*) found 294 times in ExAC as a heterozygous variant. The second was a novel *OPTN* variant (p.S174X), which causes a premature stop codon that eliminates both the UBAN domain and TBK1 phosphorylation site, as well as other important protein interaction sites (Figure 4.1). This variant was also found to be absent, even as a heterozygote, from over 70,000 controls as well as 1000 ethnically matched controls screened by Dr Gotkine's collaborators in Bethlem University. Considering that *OPTN* had already been acknowledged as an ALS causing gene and that the variant p.S174X was completely novel, it was chosen as the candidate pathogenic mutation.

All of the available DNA samples from the same family were Sanger sequenced in house by my fellow PhD student Chun Hao Wong. This confirmed that the mother (III-1) was a heterozygous carrier for p.S174X and that individuals IV-1 and IV-3 as well as IV-5 were homozygous carriers for the p.S174X variant, whereas individual IV-6 showed wild type genotype (Figure 4.2). Interestingly, IV-5 has not reported any motor neuron symptoms and has now reached the age of 54. The mean age of onset of the family is 48.5.

The sibling IV-7 has recently presented with progressive gait difficulty and limb weakness with combined UMN and LMN features suggestive of ALS. DNA was obtained and sequenced by PhD student Nada Alahmady, confirming the presence of homozygous *OPTN* variant p.S174X.

RoH analysis of the Illumina chip genotyping data confirmed that both affected siblings IV-1 and IV-3 had a block of homozygosity approximately 10Mb in size overlapping the *OPTN* gene, and that this homozygous block was not present in the heterozygous mother III-1.

4.4.3 All fibroblast lines carry the expected genotype

Primary fibroblast lines were obtained from three healthy siblings (IV-5, IV-8, IV-9) and one healthy parent (III-1, all marked with * in the pedigree in Figure 4.2 by our collaborators in the Shaare Zedek medical centre as described in section 2.5.4. Three commercially available human fibroblast control lines (described in section 2.5.4) were kindly donated to us by Professor Fiona Watt's laboratory. The *OPTN* genotype of each of these lines was confirmed by Sanger sequencing in house (Figure 4.3).

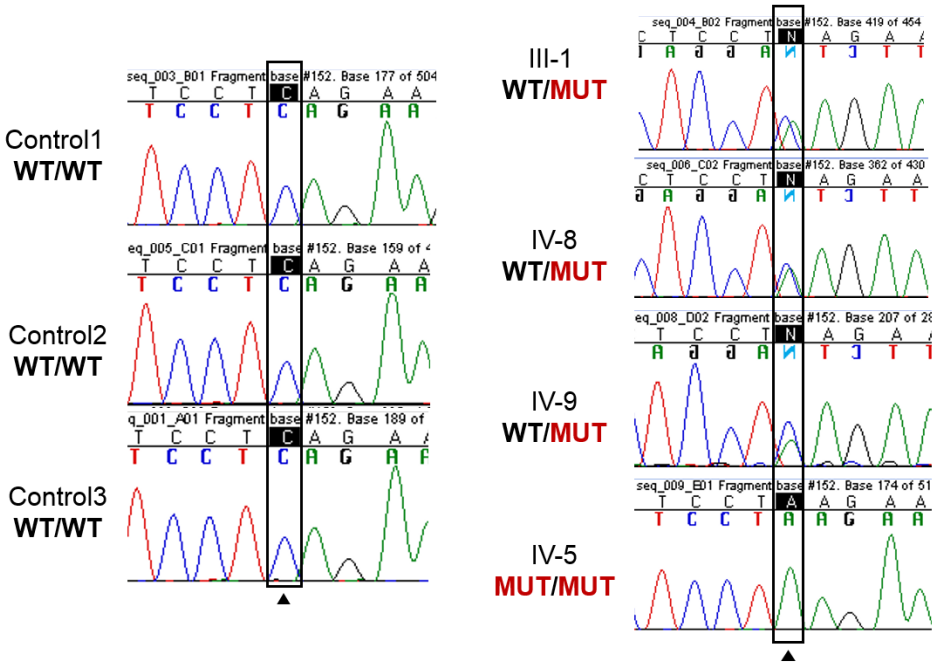


Figure 4.3 – All fibroblast lines carry the expected genotype. On the left, the genotyping for the locus of the c.521C>A, p.S174X in controls showing a wild type genotype. On the right, genotyping of the heterozygous individual derived lines. III-1, IV-8 and IV-9 show a double peak (cytosine and adenine) whereas IV-5 shows a green peak (adenine) indicating that the line carries the homozygous p.S174X change. Blue peaks indicate cytosine, red peaks indicate thymine, green peaks indicate adenine and black peaks indicate guanine.

4.4.4 *OPTN* mRNA expression is significantly reduced in p.S174X fibroblasts

In order to understand whether the expression of OPTN varied when individuals carried the heterozygous or homozygous p.S174X variant, quantitative PCR was carried out on cDNA derived from control and mutant fibroblast lines. *OPTN* mRNA levels were analysed across three biological replicates and normalised to *GAPDH*, *Ribosomal Protein L13a* (*RPL13A*) and *Succinate Dehydrogenase Complex Flavoprotein Subunit A* (*SDHA*). When grouped by genotype, there was a significant difference between the expression of *OPTN* in controls compared to heterozygous carriers (p=0.0419, Figure 4.4). Furthermore, the expression of OPTN transcript in the mutant homozygous fibroblast cell line is significantly lower when compared to any other wild type or heterozygous sample (Figure 4.4).

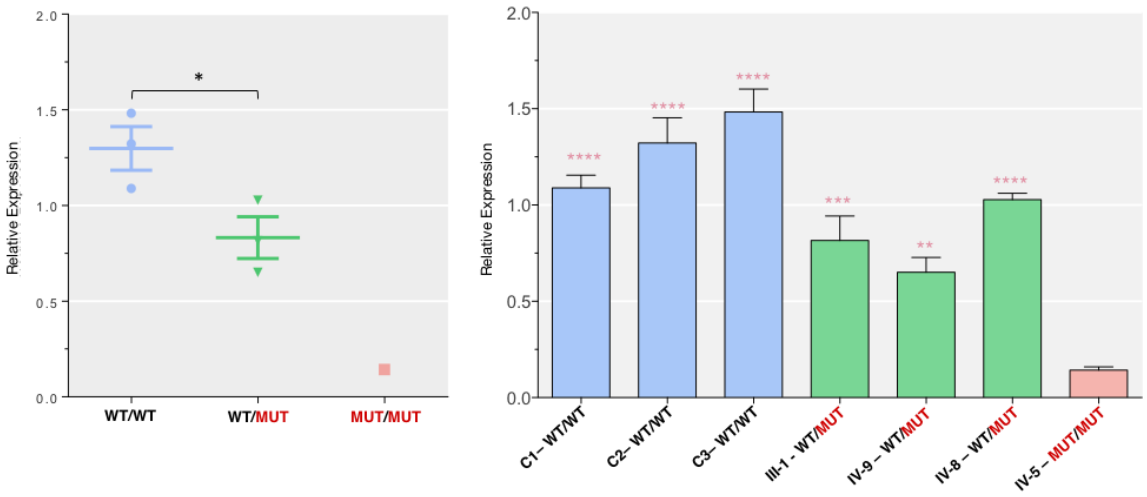


Figure 4.4 – *OPTN* expression is reduced in fibroblast harbouring the p.S174X mutation. On the left, data grouped by genotype and analysed by unpaired t-test (WT/WT vs WT/MUT two tailed p=0.0419). Error bars represent standard error of the mean (SEM). On the right, the level of *OPTN* expression per sample: controls are blue, heterozygous carriers are green and the homozygous carrier is pink. The pink stars indicate the p value derived from the comparison of IV-5 with every other individual (one way ANOVA followed by Dunnett's correction **** is ≤ 0.0001, *** is ≤ 0.001, ** ≤ 0.01, * ≤ 0.05, error bars represent SEM, n=3). Error bars represent the standard error of the mean (SEM).

4.4.5 *OPTN* variant p.S174X mRNA is degraded by nonsense mediated decay

Nonsense mediated mRNA decay (NMD) is an evolutionarily conserved mechanism that degrades mRNA containing premature stop codons (Brogna and Wen, 2009). Given that the OPTN transcript is harbouring the p.S174X we sought to determine whether its RNA might be degraded by NMD. When sequenced, the cDNA derived from the heterozygous fibroblast lines showed only a wild type genotyped, indicating that the mutated allele was not expressed at sufficient level to be detected in these cell lines (Figure 4.5). When the fibroblast lines were treated for 16 hours with 100 µg/ml cyclohexamide (CHX), an inhibitor of NMD and protein synthesis (Nickless, Bailis and You, 2017), cDNA sequence from the heterozygous lines revealed both the wild type and mutated allele, demonstrating that, the *OPTN* variant p.S174X is largely degraded through NMD. Interestingly mRNA from the homozygous mutant was detected with and without CHX, indicating that not all *OPTN* p.S174X transcripts are degraded by NMD.

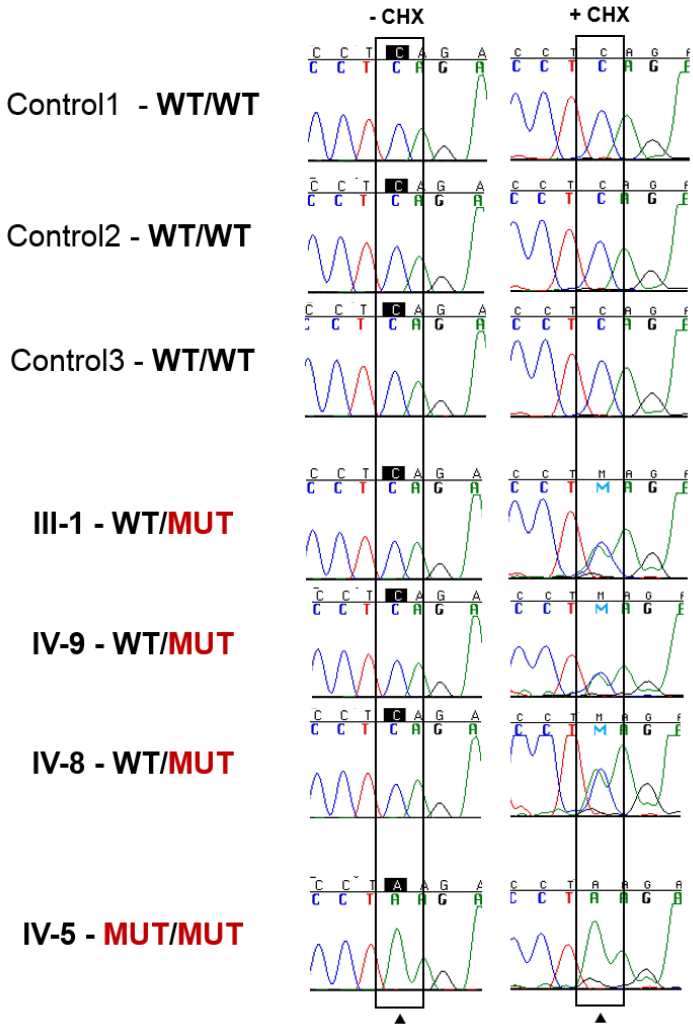


Figure 4.5 – The OPTN variant p.S174X is degraded by nonsense mediated decay. On the left, sequencing of cDNA derived by control, heterozygous and homozygous fibroblast lines, showing that the homozygous lines only present the wild type allele. On the right, sequencing of cDNA derived by control, heterozygous and homozygous fibroblast lines after treatment with 100 µg/ml CHX for 16 hours, showing a double peak in the heterozygous samples.

4.4.6 No truncated OPTN protein is detectable in OPTN p.S174X fibroblasts

All fibroblast cell lines were harvested and processed for western blotting as described in section 2.6.2. The membrane was probed for an antibody raised against the OPTN N-terminal fragment (1-14 aa) in order to determine whether any protein was translated from the OPTN p.S174X variant (expected molecular weight ~20 kDa). No OPTN protein was detected in homozygous fibroblast and no N-terminal fragment was detected in the heterozygous fibroblasts.

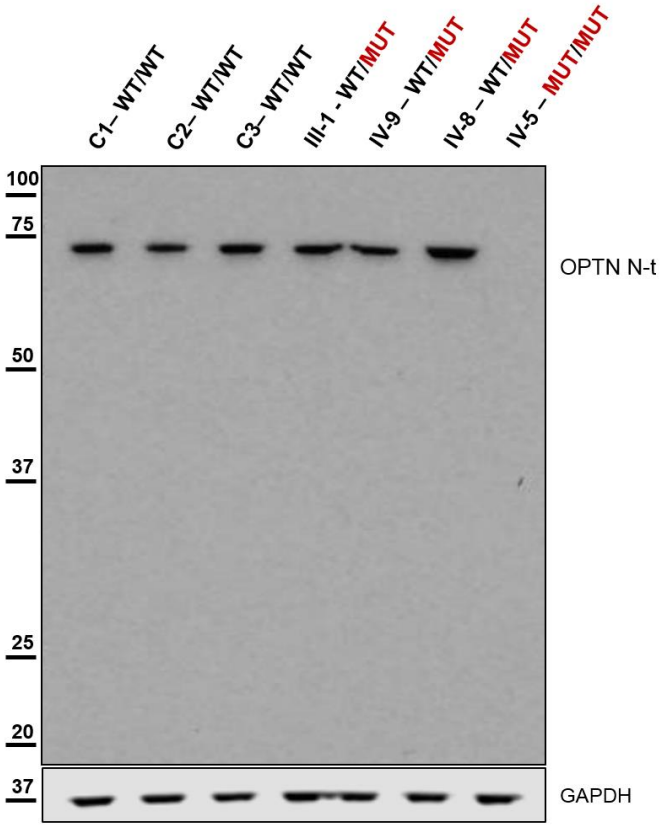


Figure 4.6 - OPTN N-terminal fragment is not expressed in IV-5. Western blot showing the presence of N-terminal OPTN (~75 kDa) in all individuals with the exception of IV-5 (MUT/MUT). p.S174X expected molecular weight ~20kDa. GAPDH shows an equal amount of protein loaded for each sample. n=3. C=Control.

4.4.7 N-terminal OPTN expression is not rescued by treatment with MG132

MG132 is a proteasome inhibitor that drastically reduces the degradation of ubiquitinated proteins (Lee and Goldberg, 1998). The OPTN p.S174X variant, if translated at all, is likely to misfold, be ubiquitin tagged and degraded by the proteasome. Therefore, if the p.S174X is translated in homozygous and heterozygous fibroblasts the truncated protein (~20 kDa) should be visible by western blot in cells treated with MG132. All fibroblast lines were, therefore, treated with 5µM MG132 or DMSO only control for 24 hours and processed for western blot as described in section 2.6.2. Immunoblot analysis showed no truncated protein for the homozygous or heterozygous samples, when treated with MG132 (Figure 4.7). Quantification of OPTN, normalised by GAPDH, revealed a significantly lower expression of OPTN in heterozygous individuals when compared to

control derived fibroblast lines, in both MG132 treated (p=0.007) and DMSO treated (p=0.0083) samples (Figure 4.7). Since a truncated protein could not be detected even following proteasome inhibition, we concluded that the OPTN p.S174X transcript is largely degraded by NMD. We were able to confirm that proteasome inhibition had occurred as the same blot was probed for p62 which was increased in all samples treated with MG132 (Figure 4.7)(Kuusisto, Suuronen and Salminen, 2001). However, we were surprised that OPTN protein did not appear to accumulate following proteasome inhibition by MG132.

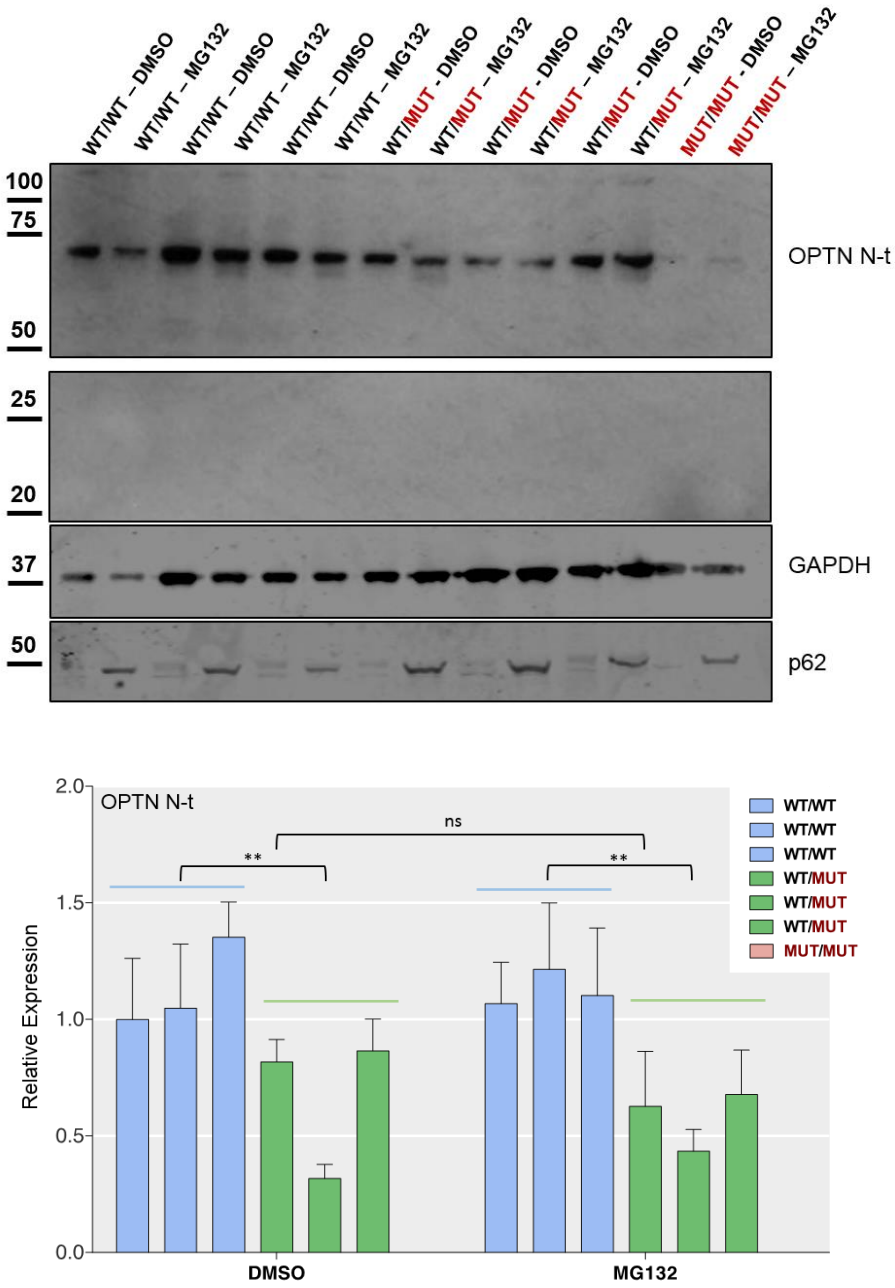


Figure 4.7 - Expression of the OPTN variant p.S174X is not rescued by treatment with MG132. On the top, western blot showing the expression of OPTN in all available fibroblast lines treated with DMSO or 5µM MG132. The expression of p.S174X is not rescued by the MG132 treatment as no truncated fragment of OPTN was detected in any of the samples. GAPDH was used as loading control and p62 was used to verify the effectiveness of the MG132 treatment. On the bottom, quantification of the amount of OPTN present in each sample normalised by GAPDH (n=4). The graph shows a significant difference between the expression level of OPTN in controls when compared to heterozygous samples (multiple t-test using Holm-Sidak method DMSOp=0.0083, MG132p=0.0072). No significant difference is observed between the expression of OPTN in cells treated with DMSO or MG132 (two way ANOVA followed by Sidak correction p=ns). Controls (WT/WT) are blue, heterozygous carriers (WT/p.S174X) are green and the homozygous carrier (p.S174X/p.S174X) is pink.

4.4.8 Heterozygous *OPTN* p.S174X, but not homozygous, fibroblasts show a modest increase in mitochondria markers

OPTN is involved in a particular subtype of selective autophagy called mitophagy (described in section 1.6.6). Impaired mitophagy causes the accumulation of defective mitochondria (Corti, Lesage and Brice, 2011), which would result in a higher expression of mitochondria markers. All primary fibroblast lines were therefore analysed by western blot and probed for the mitochondria markers TOM20 and COXIV, described in section 3.4.10. Following quantification, p.S174X heterozygous lines show a modest increase in both COXIV (unpaired t test, two tailed p=0.034) and TOM20 (unpaired t test, two tailed p=0. 026) levels when grouped by genotype and compared to controls, however no increase was detected in the homozygous line when compared individually to each other sample by one way ANOVA (Figure 4.8).

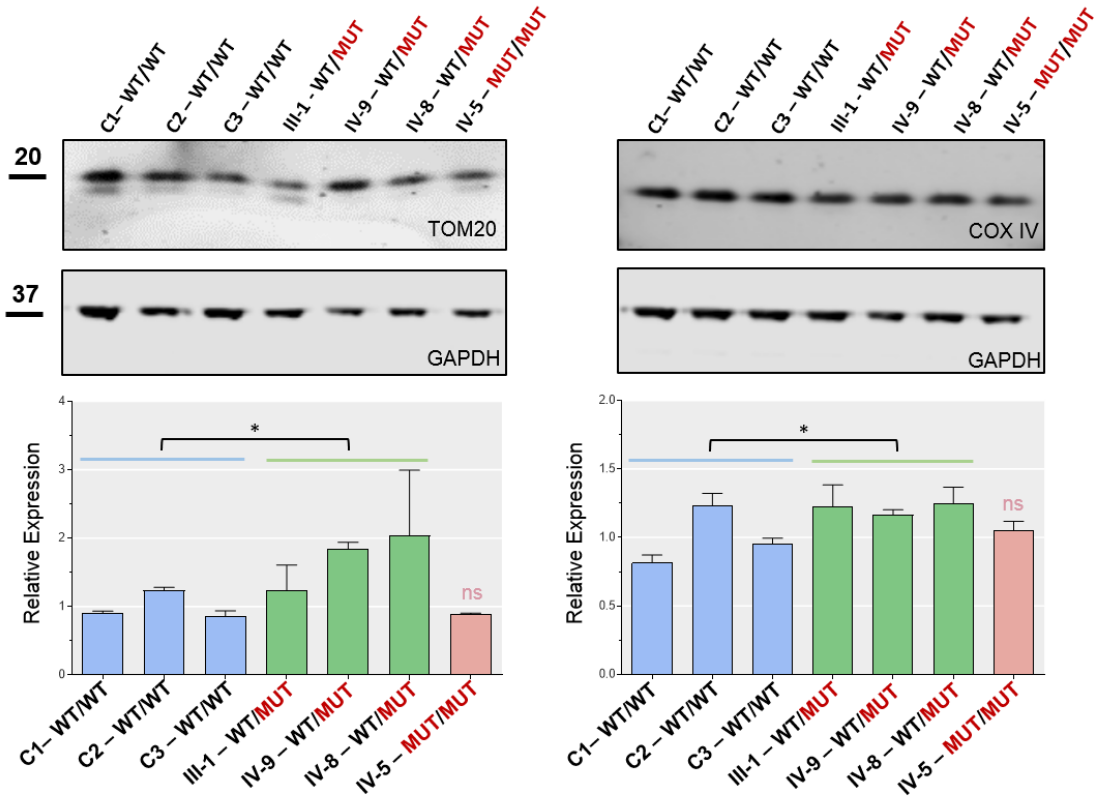


Figure 4.8 – p.S174X heterozygous fibroblast lines show a mild increase in mitochondria markers. On the left, western blot showing TOM20 expression levels in all available fibroblast lines and its quantification, normalised against GAPDH (n=3). Control and heterozygous samples were grouped by genotype and analysed by unpaired t-test (two tailed p=0.0262). The homozygous sample was compared with each

individual control and homozygous sample by one way ANOVA followed by Dunnett's correction and shows no significant difference in the expression of TOM20 (p=ns). On the right, western blot showing COXIV expression levels in all available fibroblast lines and its quantification, normalised against GAPDH (n=3). Control and heterozygous samples were grouped by genotype and analysed by unpaired t-test (two tailed p=0.0339). The homozygous sample was compared with each individual control and heterozygous line by one way ANOVA followed by Dunnett's correction and shows no significant difference in the expression of COXIV (p=ns). Error bars represent SEM. C=Control. Controls (WT/WT) are blue, heterozygous carriers (WT/p.S174X) are green and the homozygous carrier (p.S174X/p.S174X) is pink.

4.4.9 Mitochondria morphology was unchanged in p.S174X fibroblasts

In order to understand whether there were any morphological abnormalities in the fibroblast lines harbouring the p.S174X change, cells were treated with MitoTracker, fixed and stained for DAPI as described in section 2.6.1.1. The fibroblasts were then imaged (~15 cells per line, per replicate, Figure 4.9) and analysed using the ImageJ Mito-Morphology Macro as explained in section 2.6.1.1.1 (n=3). No difference was observed in the morphology of mitochondria among control, heterozygous and homozygous cells (Figure 4.10).

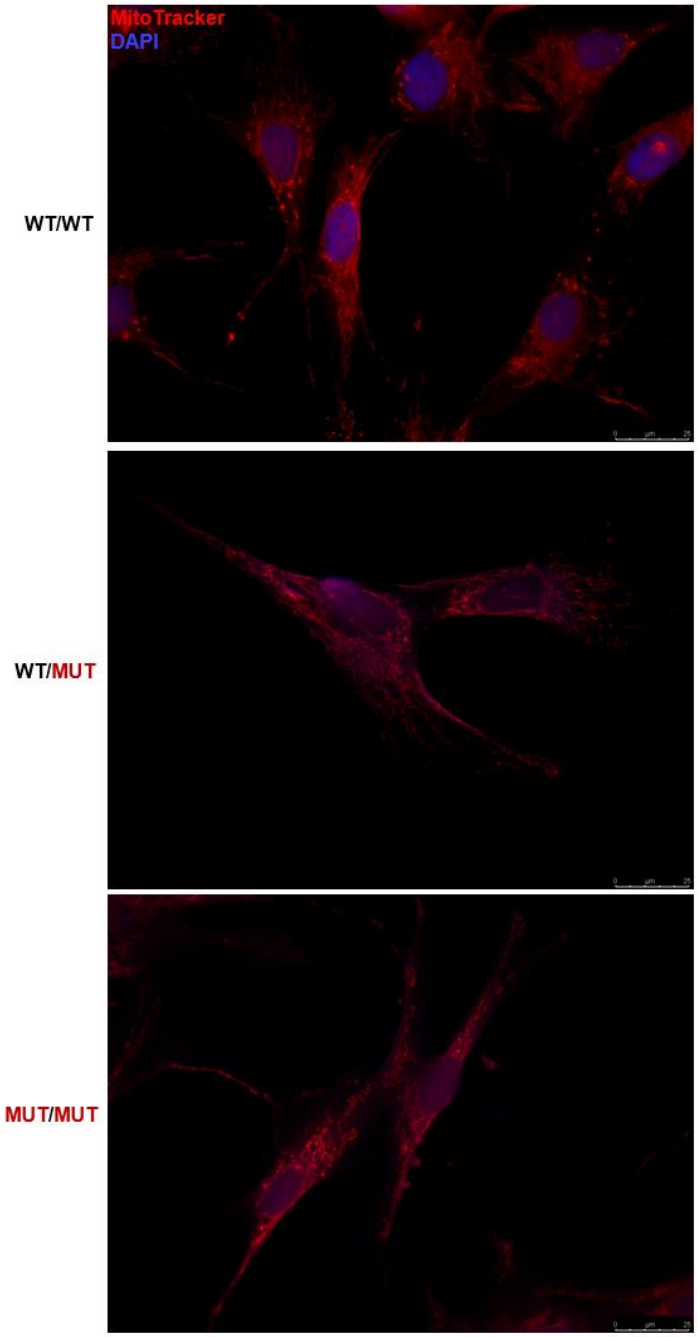


Figure 4.9 – Mitochondria in the control, heterozygous and homozygous fibroblast lines show similar morphology. Fibroblast lines were treated with MitoTracker, fixed and stained for DAPI. This figure shows one representative picture for each control, heterozygous and homozygous fibroblast lines. In red is the MitoTracker which permeated in mitochondria of the live cells. In blue is DAPI which stains the nucleus. Scale bar = 25 µm.

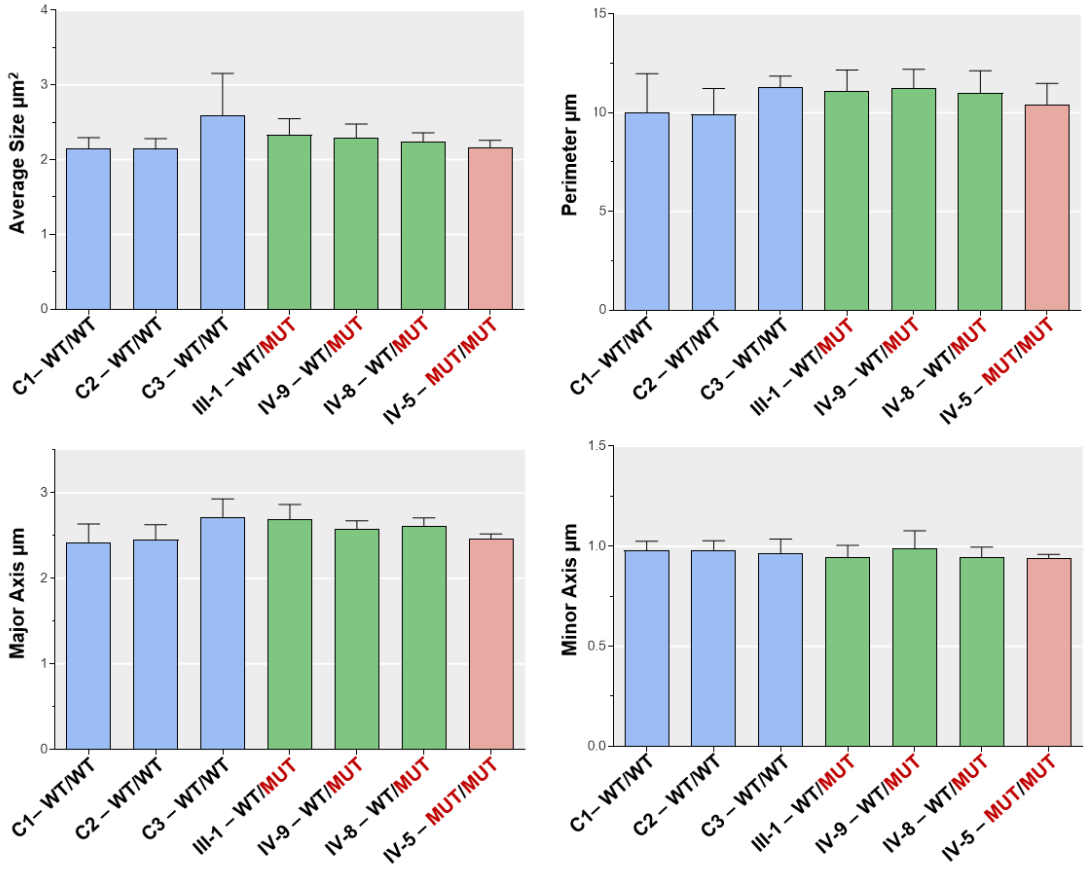


Figure 4.10 – Mitochondria morphology is similar across all fibroblast lines. Quantitative analysis of Figure 4.9 using the ImageJ Mito-Morphology Macro (Ruben, 2014). 15 cells were analysed per line and the data from three replicates quantified. No difference was detected among the different samples (analysed by one way ANOVA followed by Tukey correction, p=ns, n=3). Error bars represent SEM. C=Control. Controls (WT/WT, blue), heterozygous carriers (WT/p.S174X, green) and homozygous carrier (p.S174X/p.S174X, pink).

4.4.10 Mitochondrial membrane potential is unaffected by *OPTN* p.S174X

The mitochondrial membrane potential is fundamental for these organelles to be able to produce Adenosine triphosphate (ATP), the major energy source of the cell. For this reason, measuring mitochondrial membrane potential assay is a good index of the health of mitochondria and of the whole cell (Perry *et al.*, 2011). With this in mind, control, heterozygous and homozygous fibroblast lines were analysed using the NucleoCounter NC-3000 mitochondrial potential assay, as explained in section 2.6.8. Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) treated cells were used as a positive control. FCCP is an uncoupling agent that disrupts ATP synthesis and collapses the

mitochondrial membrane potential. As shown in Figure 4.11, no significant difference was found in the percentage of cells showing a collapsed mitochondrial membrane potential among control, heterozygous and homozygous derived fibroblasts.

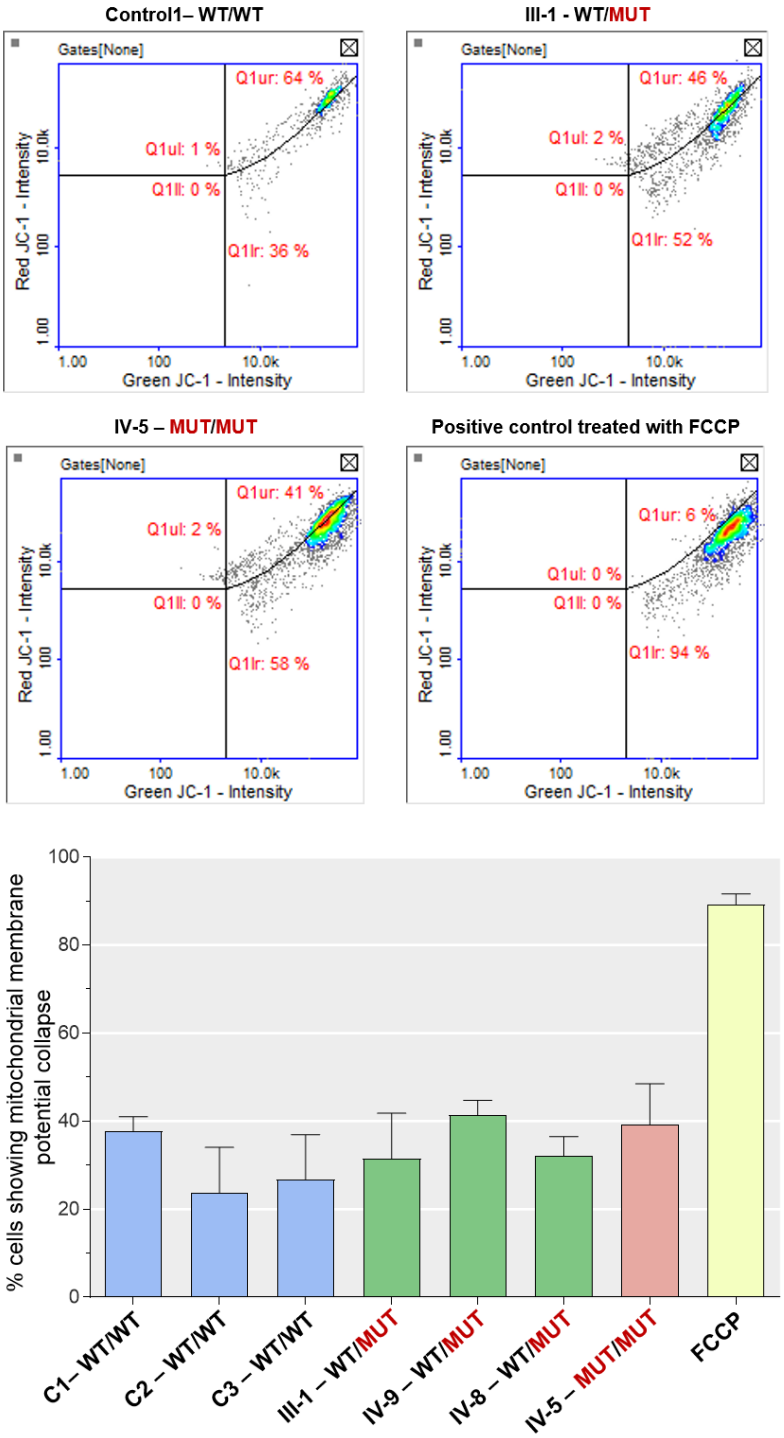


Figure 4.11 – Control, heterozygous and homozygous derived fibroblast lines do not show evidence of mitochondria membrane potential collapse. Cells were analysed using the mitochondrial potential assay for the NucleoCounter NC-3000 as decribed in section 2.6.8. On the top, an example of four two-dimensional dot plots one for each genotype and one for the positive control (fibroblasts treated with FCCP).

Red JC-1 intensity represents cells with physiological mitochondrial membrane potential, whereas Green JC-1 intensity represents cells with collapsed mitochondrial membrane potential. At the bottom, quantification of three biological replicates including the positive control. No significant difference was observed among control, heterozygous and homozygous fibroblasts (one way ANOVA followed by Tukey correction, p=0.6689, n=3). Controls (WT/WT) are blue, heterozygous carriers (WT/p.S174X) are green and the homozygous carrier (p.S174X/p.S174X) is pink, FCCP treated fibroblast line is in yellow. Error bars represent SEM.

4.4.11 OPTN p.S174X fibroblasts show a modest increase in cellular respiration

Further functional mitochondria characterisation was conducted using the Agilent Seahorse XF Cell Mito Stress Test as described in section 2.6.9. This is an automated test that records the level of mitochondria activity following the sequential application of three different drug challenges. The test initially measures the level of basal oxygen consumption (basal respiration), the cells are then treated with oligomycin, which blocks the respiration chain. The difference in the oxygen consumption between the initial phase and treatment with olygomycin, indicates the level of ATP production (Figure 4.12). Cells are then treated with FCCP which allows protons to enter mitochondria membrane unregulated and causes a peak in the mitochondrial production of oxygen, which represents the maximum respiration rate that the cell can achieve (maximal respiration, Figure 4.12). When cells are treated with antimycin A and rotenone, which block the respiration chain, cellular respiration is inhibited and cells only consume oxygen through non-mitochondrial respiration (Figure 4.12).

Our data analysis revealed that fibroblast lines heterozygous for p.S174X *OPTN* have a higher basal level of respiration (two-tailed p=0.0156), higher non-mitochondrial respiration (two-tailed p=0.0245) as well as higher ATP production (two-tailed p=0.0310), when compared to the controls (Figure 4.13). However, the heterozygous lines did not show any significant difference in their maximal respiration or spare respiratory capacity (ability of the cell to respond to an energetic need) (Figure 4.12). There was no difference in their coupling efficiency (correlation between the protons entering the mitochondrial membrane and the production of ATP calculated dividing the basal

respiration by the ATP production) when compared to controls (Figure 4.13). The fibroblast line homozygous for the *OPTN* p.S174X variant, shows a trend for defective mitochondrial function in all parameters, with the exception of the relative coupling efficiency, however this did not reach statistical significance (Figure 4.13).

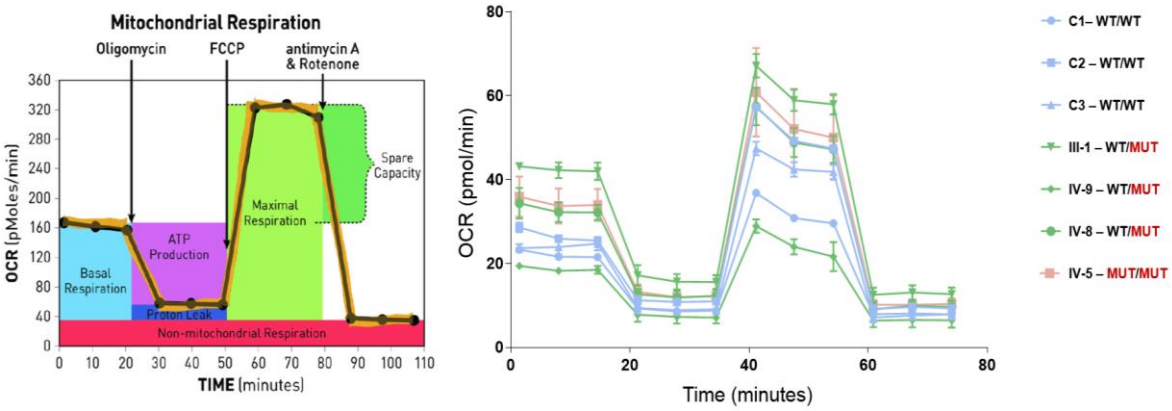


Figure 4.12 – Oxygen consumption rate (OCR) measured by the Agilent Seahorse XF Cell Mito Stress Test. On the left, an example of the OCR measurement and how it should change with the oligomycin, FCCP and antimicin A/rotenone treatments. In the initial stage, the base level of oxygen consumption is measured (basal respiration) then the oligomycin is added, which inhibits ATP synthase and blocks the respiration chains. When FCCP is added protons are free to enter the mitochondria membrane pushing the cells to their maximal respiration rate. Antimycin A and Rotenone inhibit complex I and complex III in respiration chain, shutting down mitochondria respiration. On the right, a representative example of the Agilent Seahorse XF Cell Mito Stress Test on the control, heterozygous and homozygous derived fibroblast lines. C=control.

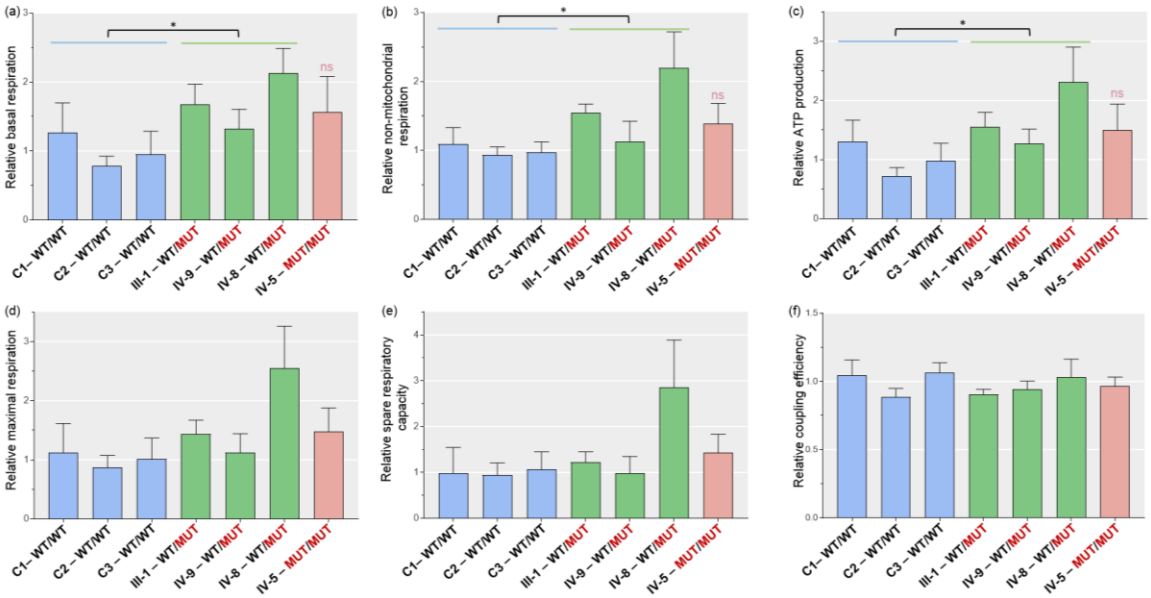


Figure 4.13 – Agilent Seahorse XF Cell Mito Stress Test analysis shows a minor increase in cell respiration in the heterozygous fibroblast lines. (a) A significantly higher basal respiration is observed in heterozygous samples when compared to controls (unpaired t-test, two tailed p=0.0156), no significant difference was detected when comparing the homozygous line to any other line (one way ANOVA followed by Dunnett's correction, p=0.2032). (b) A significantly higher non mitochondria respiration is observed in heterozygous samples when compared to controls (unpaired t-test, two tailed p=0.0245), no significant difference was detected when comparing the homozygous line to any other line (one way ANOVA followed by Dunnett's correction, p=0.0755). (c) A significantly higher level of ATP production is observed in heterozygous samples when compared to controls (unpaired t-test, two tailed p=0.0310), no significant difference was detected when comparing the homozygous line to any other line (one way ANOVA followed by Dunnett's correction, p=0.1364). (d) No difference is observed in the control and heterozygous maximal respiration (unpaired t-test, two tailed p=0.0778) or when comparing the homozygous line to any other line (one way ANOVA followed by Dunnett's correction, p=0.1711). (e) No difference is observed in the control and heterozygous spare respiratory capacity (unpaired t-test, two tailed p=0.1766) or when comparing the homozygous line to any other line (one way ANOVA followed by Dunnett's correction, p=0.1979). (f) No difference is observed in the control and heterozygous coupling efficiency (unpaired t-test, two tailed p=0.5675) or when comparing the homozygous line to any other line (one way ANOVA followed by Dunnett's correction, p=0.6181). C=control. Error bars represent SEM.

4.4.12 The autophagy marker LC3-II is not affected by OPTN p.S174X mutation

The comparison of LC3-II expression levels has been accepted as a good indicator of autophagy (Mizushima and Yoshimori, 2007). LC3 is synthesised in the form of proLC3, which is quickly processed by Atg4 into LC3-I (Kabeya, 2000). LC3-I is then converted in LC3-II through a mechanism regulated by Atg4 itself and activated by autophagy leading events such as the accumulation of reactive oxygen species during starvation (Scherz-Shouval *et al.*, 2007). This cleavage stimulates the generation of the autophagosome, which is important for the completion of the autophagy process (Klionsky, 2007; Mizushima, 2007). Because LC3-II expression is closely correlated with the number of autophagosomes present in a cell (Kabeya, 2000), an accumulation of LC3-II implies an accumulation of autophagosomes and, therefore, an impairment of autophagy (Mizushima and Yoshimori, 2007). Since OPTN is involved in the delivery of ubiquitinated cargoes to the autophagosome through LC3 binding (described in section 4.1.1.2 and 4.1.3), we measured LC3-II levels by immunoblotting in control, heterozygous and homozygous derived fibroblast lines. No difference was observed between control and p.S174X heterozygous fibroblast lines ($p=0.4277$) or by comparing p.S174X homozygous fibroblast line with any other line ($p=0.1975$), indicating no major change in the autophagosomes abundance in fibroblasts.

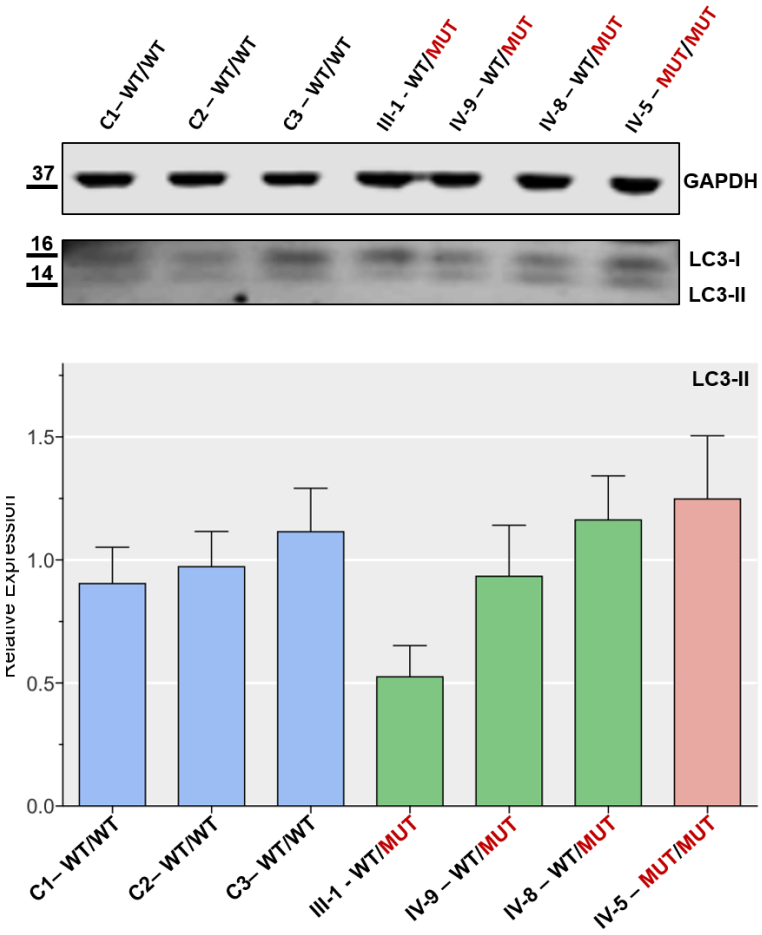


Figure 4.14 – LC3-II maintains similar expression level among control heterozygous and homozygous primary fibroblast lines. At the top, western blot showing the presence of LC3-I (~16 kDa) and LC3-II (~14 kDa) in all individuals. GAPDH shows an equal amount of protein loaded for each sample (C=Control). At the bottom, the quantification of LC3-II showing no significant difference between the control and the heterozygous group (compared by unpaired t-test, two tailed p=0.4277, n=3) or among any of the individual samples (one way ANOVA, followed by Tukey correction p=0.1795).

4.4.13 Ubiquitin K63 shows no difference in ubiquitination in OPTN p.S174X cells.

Ubiquitination of proteins is an important post transcriptional modification, which is regulated by a string of enzymatic reactions that involves ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3) (Glickman and Ciechanover, 2002). Polyubiquitination is the addition of poly-ubiquitin (poly-Ub) chains to a substrate, which are interconnected through one of the seven Lysine (K) residue of Ub. The most common types of polyubiquitinatons are the ones linked through K-48 or K-63 (Weissman, 2001). K-48 associated proteins are thought to be transported and degraded predominantly by the UPS (Xu *et al.*, 2009). On the other hand, K-63 chains

are recognised by p62 and are directed towards degradation by autophagy (Seibenhener and Babu, 2004). Furthermore, OPTN has been shown to preferably recognise K63 ubiquitin chain through its UBAN domain (Rahighi *et al.*, 2009; Gleason *et al.*, 2011). In order to determine whether OPTN deficiency would cause impaired autophagy, we probed heterozygous and homozygous OPTN p.S174X fibroblasts for K63 ubiquitin labelled proteins by immunocytochemistry, however, no striking differences were observed when compared to controls (as shown in Figure 4.15 and Figure 4.16)

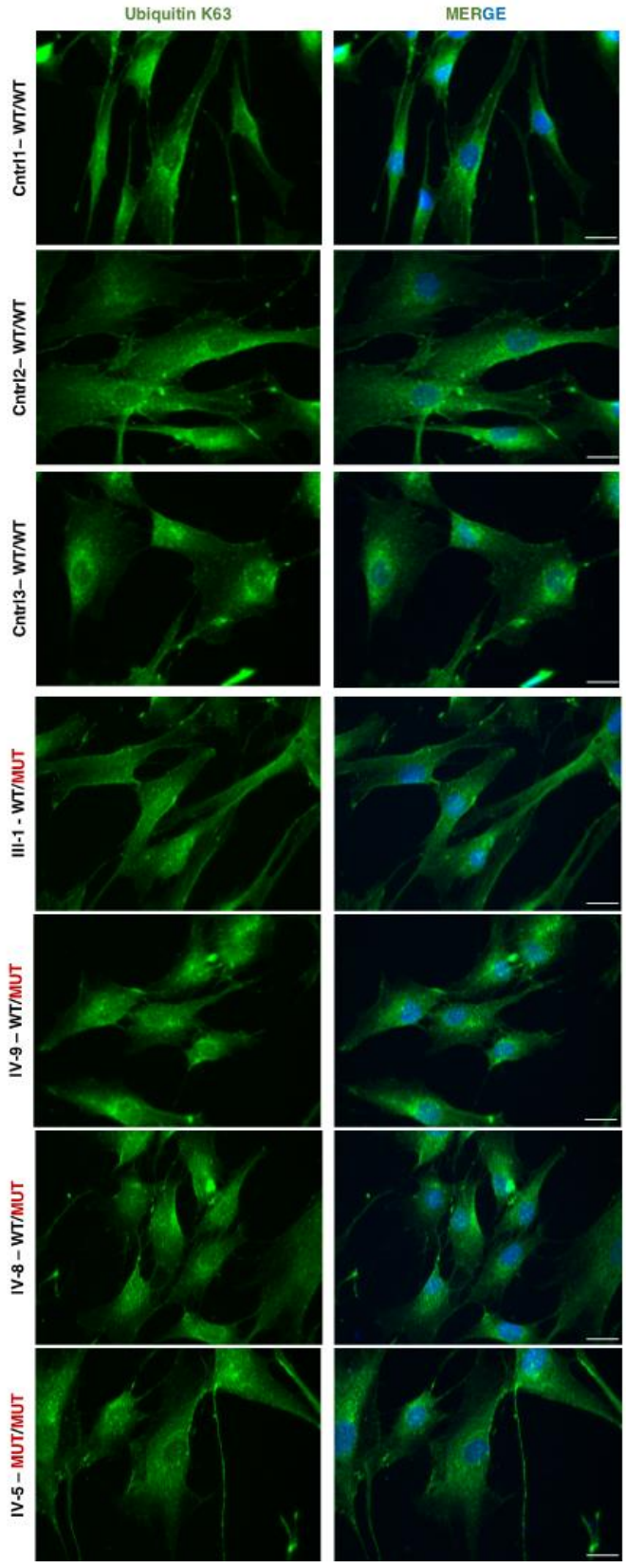


Figure 4.15 – K63 staining maintains a similar pattern across control, heterozygous and homozygous primary fibroblast lines. Representative confocal images (63x) of controls (Cntrl1-WT/WT, Cntrl2 – WT/WT, Cntrl3 – WT/W), heterozygous (III-1 – WT/MUT, IV-9 – WT/MUT, IV-8 – WT/MUT) or the homozygous (IV-5 – MUT/MUT) fibroblast line stained for K63 ubiquitin (green) and DAPI (blue). Scale bars = 20 µm.

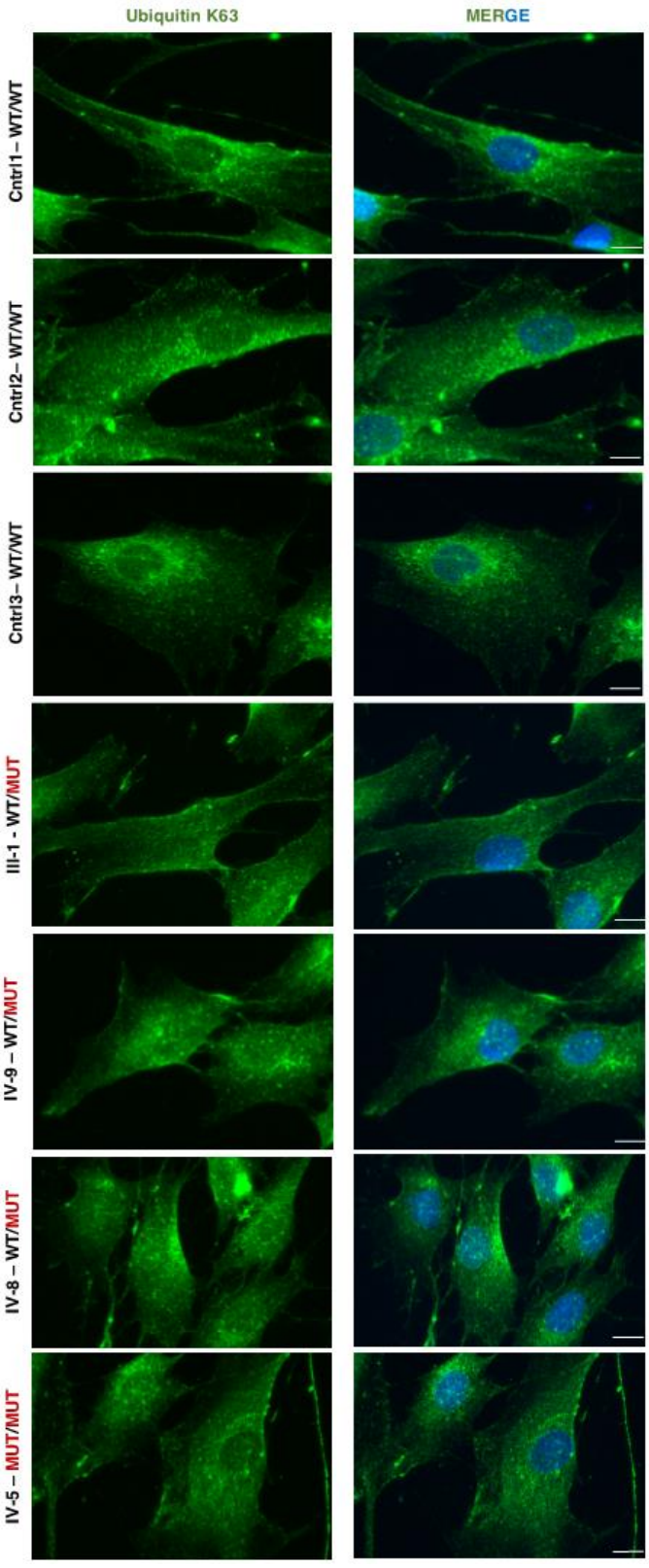


Figure 4.16 – K63 staining maintains a similar pattern across control, heterozygous and homozygous primary fibroblast lines (magnified). Higher magnification of images of controls (Cntrl1-WT/WT, Cntrl2 – WT/WT, Cntrl3 – WT/W), heterozygous (III-1 – WT/MUT, IV-9 – WT/MUT, IV-8 – WT/MUT) and the homozygous (IV-5 – MUT/MUT) fibroblast line stained for K63 ubiquitin (green) and DAPI (blue). Scale bars = 10 µm.

4.4.14 p.S174X fibroblasts do not show evidence of TDP-43 aggregation

TDP-43 aggregation is a hallmark of ALS in over 95% of the ALS cases (Peters, Ghasemi and Brown, 2015) including those carrying OPTN mutations (Maruyama *et al.*, 2010; Ito *et al.*, 2011; Pottier *et al.*, 2015). The TDP-43 aggregates are labelled with K63 and K48 ubiquitin chains and recognised by ubiquilin-1 and -2 (Neumann *et al.*, 2006; Deng *et al.*, 2011) and SQSTM1/p62 (Arai *et al.*, 2003), which will promote aggregate degradation through UPS or aggrephagy (Scotter *et al.*, 2014). In order to verify whether *OPTN* p.S174X fibroblasts showed evidence of defective proteostasis we performed immunocytochemistry for TDP-43 and p62. Occasional TDP-43 immunoreactive inclusions appeared in all fibroblast lines (red arrows) with no striking differences in their number and distribution and few appeared to colocalise with p62 (green arrows) again with no difference between control and mutant lines (Figure 4.17 and Figure 4.18).

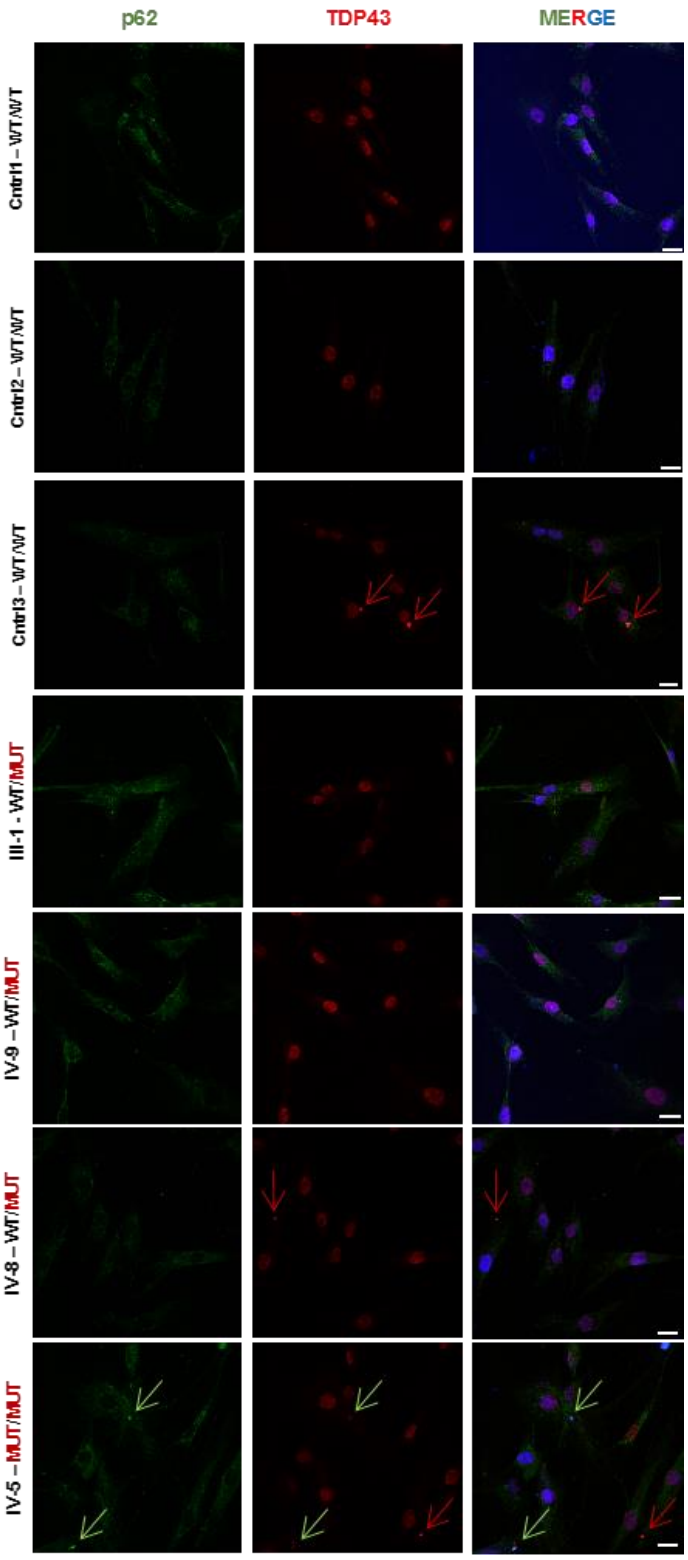


Figure 4.17 – TDP-43 staining shows mild aggregation in all fibroblast lines. Representative confocal images (40x) of controls (Cntrl1-WT/WT, Cntrl2 – WT/WT, Cntrl3 – WT/W), heterozygous (III-1 – WT/MUT, IV-9 – WT/MUT, IV-8 – WT/MUT) or the homozygous (IV-5 – MUT/MUT) fibroblast line stained for DAPI (blue), TDP-43 (red), which revealed occasional cytoplasmic inclusions (red arrows) and p62 (green) occasionally colocalising with TDP-43 inclusions (green arrows) . Scale bars = 20 µm.

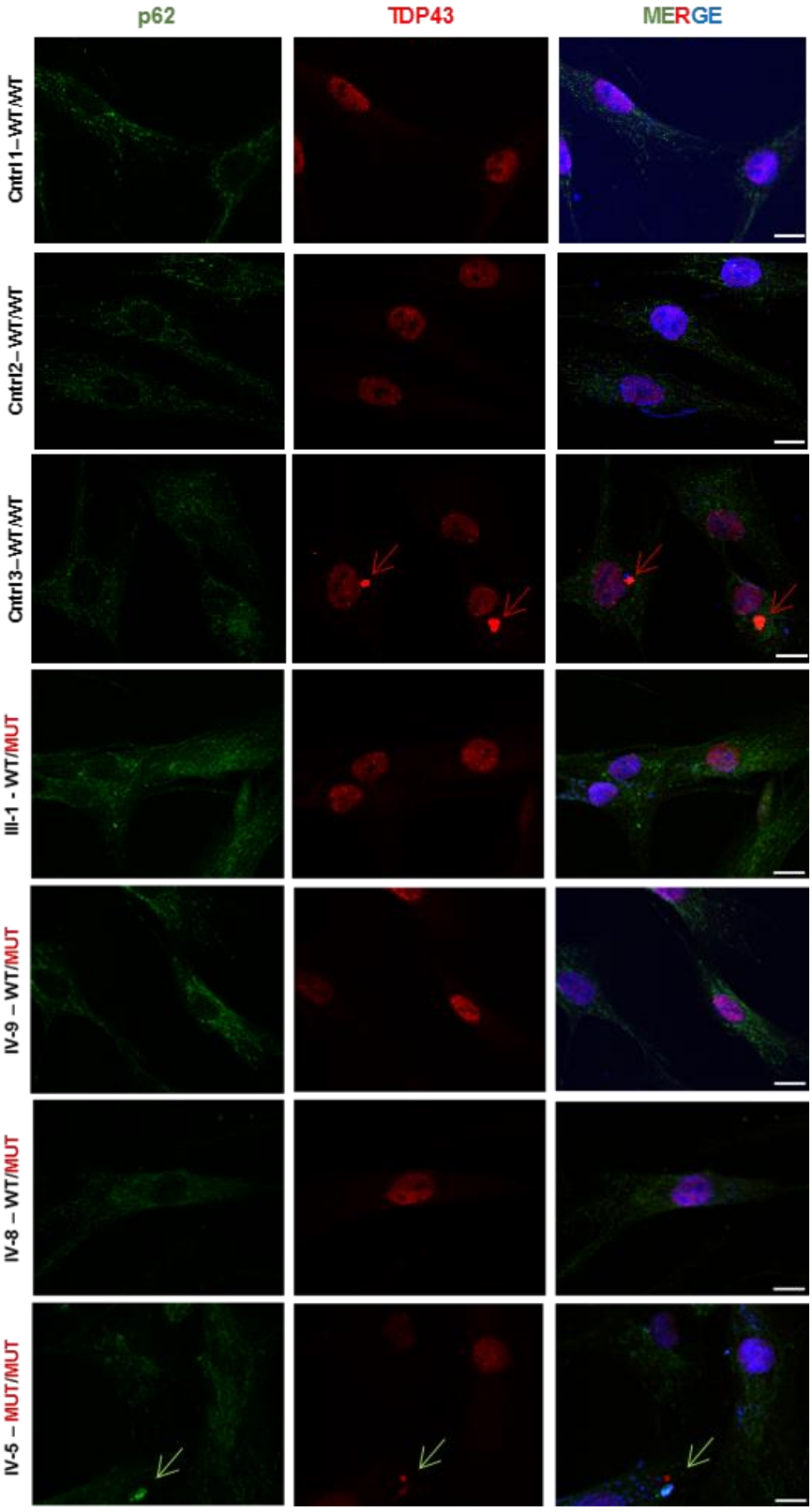


Figure 4.18 – TDP-43 staining shows mild aggregation in all fibroblast lines (magnified). Higher magnification of images of controls (Cntrl1-WT/WT, Cntrl2 – WT/WT, Cntrl3 – WT/W), heterozygous (III-1 – WT/MUT, IV-9 – WT/MUT, IV-8 – WT/MUT) or the homozygous (IV-5 – MUT/MUT) fibroblast line stained for DAPI (blue), TDP-43 (red), which revealed occasional cytoplasmic inclusions (red arrows) and p62 (green) occasionally colocalising with TDP-43 inclusions (green arrows). Scale bars = 10 µm.

4.5 Discussion

This study shows the genetic and functional characterisation of a novel *OPTN* variant (p.S174X) found by shared region of homozygosity and exome sequencing of a consanguineous Palestinian family affected by an aggressive ALS phenotype (Figure 4.2). All four affected siblings carried the *OPTN* p.S174X non-sense mutation and their heterozygous parents and siblings were unaffected. This variant was absent from >70,000 individuals of European ancestry and 1000 ethnically matched Palestinian controls, showing that this variant is rare if not absent in these two populations.

Investigating consanguineous families is an efficient technique to discover novel recessive mutations that can occur as homozygous variants and therefore be easier to identify. This method has been successfully used in ALS gene discovery and investigation (Yang *et al.*, 2001; Maruyama *et al.*, 2010).

Through an international collaboration with Dr Marc Gotkine at The Hebrew University Hadassah Medical School in Jerusalem, we obtained fibroblast lines from four members of a consanguineous Palestinian family (Figure 4.2). Three healthy individuals carried the *OPTN* p.S174X variant as heterozygotes and one unaffected individual was homozygous. In order to verify whether these fibroblast lines showed any phenotypic abnormalities, they were compared to three commercially available Caucasian controls carrying the WT *OPTN* allele (Figure 4.3). One limitation of this study is the different ethnic origin of the members of the family and controls. Indeed, it is possible that some of the differences here observed could be due to a different genetic background. It would be, therefore, interesting to repeat some of the assays presented here using ethnically matched controls.

Quantitative PCR of these lines showed a significant decrease of OPTN mRNA in the heterozygous when compared to control fibroblasts and a severe loss of OPTN transcripts in the homozygous cells (Figure 4.4). I demonstrated that the decrease in OPTN mRNA was due to the degradation of the mutated transcript by nonsense

mediated decay (NMD) as cDNA sequencing from heterozygous fibroblast lines detected only a WT genotype and treatment with CHX (an NMD inhibitor) rescued the heterozygous genotype (Figure 4.5). This finding has previously been observed by Maruyama and colleagues who showed that the level of OPTN expression in patients homozygous for the p.Q398X variant was dramatically diminished and was rescued by CHX treatment (Maruyama *et al.*, 2010).

The absence of a truncated OPTN protein derived from the p.S174X allele was confirmed by western blot analysis (Figure 4.6). A similar result was also reported by Maruyama and colleagues who observed loss of OPTN expression in lymphocyte cell lines derived from patients homozygous for the *OPTN* p.Q398X variant (Maruyama *et al.*, 2010). Here we report that heterozygous p.S174X individuals also have a reduced expression of OPTN when compared to controls. Furthermore, when the proteasome was inhibited, the expression of the truncated form of OPTN was not detected confirming NMD of the mutant transcript (Figure 4.7). When treated with MG132 control, heterozygous and homozygous fibroblast lines show an accumulation of p62, however, no difference was found in the levels of OPTN between DMSO and MG132 treated cells. This result is consistent with what was observed in neuroblastoma cells (Shen *et al.*, 2015) however, it is not consistent with what was shown in HEK293 cells (Mao *et al.*, 2017).

OPTN has an important role in the mitophagy pathway and the lack of OPTN has been previously correlated with higher expression of mitochondria markers such as TOM20 (Wong and Holzbaur, 2014). For this reason, mitochondria abundance, function and morphology were analysed in the fibroblast lines. Although the expression of mitochondria markers COXIV and TOM20 was found to be marginally higher in heterozygous samples compared to controls (Figure 4.8), there does not seem to be any morphological difference in their mitochondria using mitotracker stain (Figure 4.10). Heterozygous fibroblast lines also showed a higher rate of ATP production, as well as basal and non-mitochondrial respiration when compared to the controls, however, they

did not show a difference in the maximal respiration, spare respiratory capacity and coupling efficiency and the mitochondria membrane potential was unchanged (Figure 4.11 and Figure **4.13**). One explanation is that damaged mitochondria might be degraded more slowly in these cells compared to controls, which would explain why mitochondria were more abundant, as measured by TOM20 or COXIV blots (Figure 4.8). These mitochondria might retain some of their functions boosting the ATP production but might not be able to perform to their maximum respiratory function when challenged in the Seahorse assay, which would explain why there was no difference in the maximal respiration parameters (Figure 4.13). These cells also show higher non-mitochondrial respiration, which may imply a compensatory mechanism to support the fully functional mitochondria.

Curiously, I observed no difference in mitophagy and autophagy markers when comparing the p.S174X homozygous fibroblasts to controls. This could be because only one homozygous p.S174X individual was available, which limited the power of the study and it is indeed possible a larger number of homozygous individuals would have shown a difference. Another possible explanation is that homozygous p.S174X fibroblasts have developed without any OPTN and may have generated a compensatory mitophagy mechanism that might fail with age. Lastly, it is also possible that given that individual IV-5 was 54 at the time of the study and has not manifested symptoms of ALS that she individual has an unknown protective mechanism not shared by her affected siblings.

It has been previously reported that OPTN silencing as well as the expression of p.Q398X or p.E478G in neuroblastoma cell lines is sufficient to create a disruption of the autophagosome-lysosome fusion, leading to an accumulation of LC3-II (Shen *et al.*, 2015; Sundaramoorthy *et al.*, 2015). However, in this study LC3-II quantification in mutant lines showed no expression difference from controls (Figure 4.14), providing no evidence of major disruption of the autophagosome-lysosome fusion. A qualitative staining of ubiquitin K63 has also shown no striking differences in the distribution of K63 ubiquitin chains (Figure 4.15 and Figure 4.16), which implies no accumulation of

ubiquitinated material in these cells. These observations suggest that the lack of OPTN in fibroblasts does not significantly disrupt the autophagy machinery. However, these data were collected in mitotically active culture fibroblasts growing in optimal conditions. It is possible that homozygous and heterozygous fibroblast lines would show a disruption of the autophagy pathway when stressed with inhibitors of autophagy or other cellular stressors. It is also possible that defects in autophagy are most marked in post-mitotic motor neurons which may be more susceptible to autophagic defects as they are the cells that degenerate in ALS and skin fibroblasts are not know to be affected in OPTN deficient patients.

As ALS patients harbouring mutations in OPTN have previously been shown to accumulate neuronal cytoplasmic TDP-43 inclusions on pathological examination (Maruyama *et al.*, 2010; Pottier *et al.*, 2015), I stained the fibroblast lines for TDP-43 and p62, in order to determine whether they showed any pathological changes. TDP-43 aggregates were occasionally found in all of the cell lines and no striking difference were observed. Furthermore, no difference was observed in the colocaliation of p62 and TDP-43 inclusions in any of the analysed cells. Once again, stressing the cells could reveal a phenotype that appears only subtle when cells are growing in a physiological-like state. Unfortunately, due to time constrains, this study only shows a qualitative analysis of these aggregates in one replicate. It would be worthwhile analysing these data quantitatively across three biological replicates to determine whether there is a statistically significance difference.

4.6 Conclusions

In summary, this study identified a novel *OPTN* truncation mutation (p.S174X) that causes an aggressive form of ALS which is inherited in an autosomal recessive fashion. The expression of this variant is inhibited by nonsense mediated decay, therefore, individuals homozygous for the p.S174X mutation completely lose OPTN protein expression. OPTN is involved in many important cellular pathways but loss of autophagy

and mitophagy function has been hypothesised as the likely neurodegenerative mechanism (Maruyama *et al.*, 2010). Interestingly, this study did not present any striking mitophagy or autophagy phenotype, which have been shown to be altered in OPTN knock out cellular models before (Wong and Holzbaur, 2014; Shen *et al.*, 2015; Sundaramoorthy *et al.*, 2015). However, these studies have been conducted in HeLa cells, cervical cancer derived cell line, Neuro2A cells, a mouse neuroblastoma cell line, and NSC-34, a hybrid cell line obtained from the fusion of embryonic murine spinal cord and mouse neuroblastoma cells. These are all immortalised cell lines with a high replication rate, which are very different from patient primary fibroblasts used in our study. Indeed, primary fibroblasts have a very slow replication rate and a slow metabolism, which may make any subtle phenotype difficult to detect. ALS is a motor neuron disease that does not show any manifestation in the skin, however, it is valuable to study patient derived cells which carry their genetic information in the correct genomic and epigenetic context. iPSCs have recently been developed from the homozygous fibroblast line, which will be differentiated in motor neurons and investigated further in order to understand whether a motor neuron specific phenotype does occur.

Chapter 5 – Genetic and functional characterisation of *MATR3* ALS-linked variants

5.1 Background

Matrin 3 (encoded by *MATR3*) was associated with ALS in 2014 when an exome sequencing study identified four candidate heterozygous variants in a cohort of 205 fALS cases, three of which segregated with disease (Johnson *et al.*, 2014). Mutations in *MATR3* have also been associated with autosomal dominant, distal, asymmetrical myopathy with vocal cord paralysis (Feit *et al.*, 1998; Senderek *et al.*, 2009). Interestingly, one of the family identified by Johnson and colleagues that carries the p.S85C *MATR3* mutation, was initially diagnosed with distal myopathy and lately with ALS (Johnson *et al.*, 2014), other studies have also reported distal myopathy in patients harbouring the p.S85C *MATR3* variant (Müller *et al.*, 2014; Palmio *et al.*, 2015). Since 2014, 15 different *MATR3* variants (Table 5.1) have been published in screening of ~2000 ALS patients from 7 different countries (US, Australia, France, Italy, Taiwan, French Canada and China), with a mutation frequency of 0.8% in ALS cases (Fifita *et al.*, 2014; Millecamps *et al.*, 2014; Lin *et al.*, 2015; Origone *et al.*, 2015; Leblond *et al.*, 2016; Xu *et al.*, 2016; Marangi *et al.*, 2017). These patients present with a mean age of onset of ~57 and show a large range of survival (2-12 years), most had sporadic disease and interestingly very few had features of dementia (Johnson *et al.*, 2014; Lin *et al.*, 2015; Origone *et al.*, 2015; Leblond *et al.*, 2016; Marangi *et al.*, 2017) (Table 5.1).

In the original publication by Johnson and colleagues, post mortem human tissue from spinal cord of MATR3-ALS patients carrying the p.F115C variant, showed intense MATR3 nuclear staining compared to lighter MATR3 staining in neurologically normal controls (Johnson *et al.*, 2014). Furthermore, analysis of skeletal muscle tissue in these patients, revealed that TDP43 and MATR3 mislocalise to the sarcoplasm, the cytoplasm of skeletal muscle cells, aggregating to form small cytosolic inclusions. Interestingly, a subsequent study on a skeletal muscle biopsy, obtained from a patient carrying a

different *MATR3* variant (p.Q66K), has shown no significant difference in the localisation of MATR3 between the patient and a control (Marangi *et al.*, 2017).

Mutation[a]	Exon	AA Change	Cases	Affected Domain	Frequency in ExAC	Gender	Phenotype	Site of onset	Age of onset	Survival (years)	Origin	Familial	Reference
c.48+1G>T	5	-	1	-	1	M	-	DUL	63	1.8	Canada	N	(Leblond *et al.*, 2016)
c.196C>A	5	p.Gln66Lys	1	-	0	M	LMN	R	51	2	Italy	N	(Marangi *et al.*, 2017)
c.214G>A	5	p.Ala72Thr	1	-	0	M	Classic	L	43	11	China	N	(Lin *et al.*, 2015)
c.254C<G	5	p.Ser85Cys	6 affected relatives	-	0	-	Classic	3LL,2B,1UL	44,42,33,47,44,49	21,15,32,16,16,1all patients are alive	USA	Y	(Johnson *et al.*, 2014)
c.344T>G	5	p.Phe115Cys	5 affected relatives	-	1	2M,2F,1-	-	3UL,2LL	50,52,57,63,70	-	-	Y	(Johnson *et al.*, 2014)
c.-339+2T>A	5	-	1	-	0	M	-	B	79	-	Canada	N	(Leblond *et al.*, 2016)
C439A>T	5	p.Arg147Trp	1	-	0	M	Classic	L	43	9	Italy	N	(Origone *et al.*, 2015)
c.457G>T	5	p.Gly153Cys	2	-	0	M,M	UMN-D	LL,UL	48,55	15,7	Italy	Y,N	(Marangi *et al.*, 2017)
c.460A>G	5	p.Pro154Ser	1	-	0	-	-	UL	59	-	Asia	N	(Johnson *et al.*, 2014)
c.1180G>A	9	p.Val394Met	1	RRM1	0	M	-	B	58	-	Canada	N	(Leblond *et al.*, 2016)
c.1829C>T	15	p.Ser610Phe	1	-	0	F	-	UL	48	-	China	N	(Xu *et al.*, 2016)
c.1864A>G	15	p.Thr622Ala	2 first cousins	-	0	F,F	Classic, -	UL,L	60,62	2.75, -	Italian	Y	(Johnson *et al.*, 2014)
c.1991A>C	15	p.Glu664Ala	1	-	36	F	Classic	B	64	2	Italy	N	(Marangi *et al.*, 2017)
c.2120C>T	15	p.S707L	1	NLS	0	M	UMN-D/FTD	B	57	12	Italian	-	(Marangi *et al.*, 2017)
c.2360A>G	16	p.N787S	1	-	46	M	Classic	LL	51	2	Italian	N	(Marangi *et al.*, 2017)

Table 5.1 – All reported MATR3 associated ALS variants and their clinical characteristics. [a] Mutation nomenclature as recommended by the Human Genome Variation society www.hgvs.org, utilising +1 as the A of the initiator Met codon, translation start site. DUL=Distal upper limb, R=respiratory, L=limbs, LL=lower limbs, UL=upper limbs. UMN-D=upper motor neuron dominant

MATR3 encodes for a highly conserved protein, Matrin 3 (MATR3), involved in RNA processing. It localises to the nucleus and is part of the nuclear matrix, a system that supports the nucleus structurally and functionally. MATR3 contains a nuclear export signal (NES), two zinc finger domains (ZF), two tandem RNA recognition motifs (RRM1, RRM2), a nuclear localisation signal (NLS) and a membrane retention signal (MRS) as shown in Figure 5.1 (Hibino et al., 2006). MATR3 has been reported to be involved in hyper edited mRNA retention to the nucleus, preventing its translation (Xu *et al.*, 2011), DNA repair (Rowland, Hneider and Shneider, 2001), alternative splicing (Coelho *et al.*, 2015) and NMDA-mediated neurotoxicity (Giordano *et al.*, 2005). MATR3 is also found to be phosphorylated by the nuclear protein kinase Ataxia Telangiectasia Mutated (ATM) in response to double strand break in DNA, and to cooperate with SFPQ and NONO in DNA damage repair (Salton *et al.*, 2010). Furthermore, MATR3 expression has been found to be reduced in the CNS in foetal Down's Syndrome (Bimpaki *et al.*, 2010). A recent study has highlighted that MATR3 is expressed at low levels in the muscle and spinal cord of mice, and in patients with ALS and distal myopathy, compared to the rest of the CNS and other tissues (Rayaprolu *et al.*, 2016). Furthermore, overexpression of human wild type MATR3 in mice causes a motor phenotype which shows incomplete penetrance as well as clear evidence of pathology in muscle tissue (Moloney *et al.*, 2016).

Little functional characterisation has been carried out on ALS-linked *MATR3* variants. Johnson and colleagues have shown that MATR3 associated with TDP43 in an RNA-dependent manner and that the p.S85C *MATR3* mutant binding to TDP-43 is stronger than WT ($p<0.01$). A different study reported that overexpression of wild type and mutant MATR3 (p.S85C, p.F115C, p.P154S and p.T622A) does not alter its cellular localisation in Chinese hamster ovary (CHO) and human neuroglioma cells (Gallego-Iradi *et al.*, 2015). Further investigation of MATR3 mechanism of pathogenicity is necessary in order to understand its role in ALS.

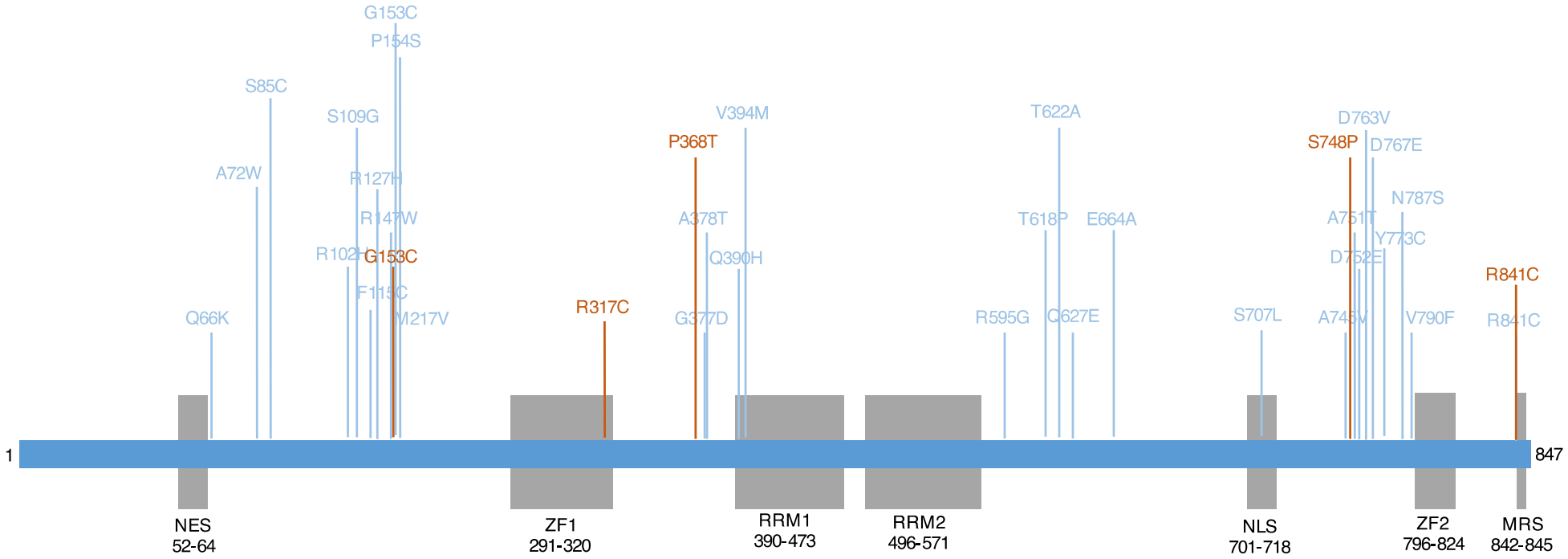


Figure 5.1 – Matrin 3 protein structure (according to Hibino et al. 2006). The figure shows all the mutations annotated in ALSdb (found ≤ 13 times in ExAC) and reported in literature to date (in light blue) as well as mutations found by our exome sequencing project (in maroon). NES=nuclear export signal, ZF=zinc finger, RRM=RNA recognition domain, NLS= nuclear localisation signal, MRS=membrane retention signal. Transcript NM_199189, ENST000003610.

5.1.1 Exome sequencing study

WES was performed on a cohort of 932 patient samples primarily of European ancestry of which 757 were index cases and 175 were affected relatives. Any sample positive for mutations in known ALS genes (eg. *SOD1*, *TARDBP*, *FUS*, *PFN1*, *UBQLN2*, *OPTN*, *VCP*, and *ANG*) or for the *C9ORF72* hexanucleotide expansion (Dejesus-hernandez *et al.*, 2011) were excluded from further analysis, resulting in a cohort of 699 index samples from eleven countries (UK, USA, Italy, Spain, Canada, Germany, Netherlands, Belgium, New Zealand, Israel and Ireland, Table 2.1). The DNA extraction and exome capture were performed by previous lab members Athina Soraya Gkazi and Jack Miller and sequencing was outsourced mainly to the Genomic Core Facility at Guy's Hospital. The data analysis was performed by my colleague bioinformatician Simon Topp.

Five novel and/or potentially damaging protein changing variants were identified in *MATR3* (Table 5.3). When this project was initiated, the ExAC (http://exac.broadinstitute.org) and GnomAD (http://gnomad.broadinstitute.org/) databases had not yet been made available. Variants were, therefore, filtered, as explained in section 2.2, against neurologically normal controls in the ESP (http://evs.gs.washington.edu/EVS), UK10K (www.uk10k.org) and a 1000 genomes (http://www.internationalgenome.org/) cohort accounting for a total of 12,584 control samples. When the ExAC and GnomAD genomes data was released the analysis was adjusted to include the data from ~60,000 and ~15,000 additional control samples respectively, reaching a control number > 80,000 for most loci. Although some variants were no longer novel they remained extremely rare (i.e. p.R317C and p.R841C).

All of the rare candidate variants found in this study are missense heterozygous variants. This is in accordance with the literature as no nonsense or homozygous *MATR3* variants have been associated with ALS to date. Four of the five variants have been found at least once in exome databases, however p.G153C is the only variant that maintained its

novelty after the addition of the ExAC and GenomAD data and it has also been recently described in two unrelated ALS cases (Marangi *et al.*, 2017).

5.2 Aims of the project

The aim of this project was to functionally investigate a subset of variants likely to be damaging as well as to determine *MATR3* variant frequency in a cohort of 174 apparently sporadic ALS cases. This project ultimately aimed to determine the contribution of *MATR3* variants to motor neuron degeneration in ALS.

5.3 Materials and methods

Most of the materials and methods used in this section have been previously described in Chapter 2. Techniques specific to this chapter are explained below.

5.3.1 Genotyping of patients carrying the *MATR3* p.R841C variant

Patient DNA was amplified and sequenced as described in section 2.3 using the primers below (Table 5.2).

	Forward	Reverse
Amplification and sequencing	TGCTGCATTTCTCTTAGGTGAC	CCAAAACGGGTTCAAACTCCA

Table 5.2 –Primers used genotyping of *MATR3* p.R841C variant. Primers were designed using Primer 3 as described in section 2.3.1. Sequences are represented 5' – 3'.

5.4 Results

5.4.1 Genetic screening for *MATR3* variants

In order to determine *MATR3* variant frequency in our sporadic cohort, I performed Sanger sequencing on six out of the fourteen coding exons of MATR3 in 174 apparently sporadic ALS cases and eight exons were sequenced by an MSc student Tegan Fletcher under my supervision. Due to prolonged technical issues sequencing of exons 11, 13

and 15 was not possible. No *MATR3* variants were identified in DNA from the 174 sALS cases.

5.4.2 Selection of subset of variants

Five *MATR3* variants were identified in our cohort of 699 familial ALS cases (0.7%). When this study was initiated we had access to a restricted subset of exomes from 1000 genomes, EVS and UK10K databases (n=12,584). Three of our variants (p.G153C, p.R317C and p.R841C) were considered novel and consistently predicted to be damaging by the algorithms SIFT, Polyphen2-hdiv and Polyphen2-hvar. p.R841C was found to be present in samples from two unrelated cases. However, the genomic locus chr5:139329372, which corresponds to the c.2521 nucleotide on exon 18 of *MATR3* that is mutated in the p.R841C variant, had no coverage in EVS database decreasing the number of individuals sequenced for this region to ~6,000. Our collaborator Dr Nicola Ticozzi sequenced exon 18 in 3,596 controls and none carried the p.R841C variant. p.R841C was later found in a larger cohort of exomes in ExAC database, an up to date study of its enrichment in the available ALS population is presented in section 5.4.3.

Variant[a]	Exon	Predicted aa change	Affected individuals	Affected domain	Frequency in controls	Damage prediction	Gender	Clinical Diagnosis	Site of onset	Age of Onset (years)	Disease Duration (Months)	Country of Origin	Reference
c.457G>T	5	p.Gly153Cys	1	-	0	Damaging	M	ALS	Upper Limb	51	12	Italy	(Marangi *et al.*, 2017)
c.949C>T	6	p.Arg317Cys	1	ZF	1	Damaging	M	ALS	Spinal	44	72	Italy	-
c.1102C>A	8	p.Pro368Thr	1	-	2	Damaging	F	ALS	-	49	12	UK	-
c.2242T>C	16	p.Ser748Pro	1	-	4	Benign	M	ALS	-	-	-	UK (Iraq Jew)	-
c.2521C>T	18	P.Arg841Cys (ALS0079)	2	-	19	Damaging	M,F	ALS	-	-	-	UK	ALSdb

Table 5.3 – Description of the nonsynonymous variants identified in *MATR3* and their clinical characteristics. Damage prediction was verified using the cumulative score of 20 pathogenicity prediction algorithms (2.2.3). [a] Mutation nomenclature as recommended by the Human Genome Variation society www.hgvs.org, utlizing +1 as the A of the initiator Met codon, translation start site.

DNA samples from the two unrelated British patients carrying p.R841C were Sanger sequenced in house and this detected the variant to be heterozygous in both cases (double peak where red stands for thymine and blue for cytosine) and absent from the control sample (Figure 5.2). Unfortunately, we did not have access to any other DNA sample from the other individuals harbouring *MATR3* nonsynonymous variants in our cohort, as they were initially exome sequenced by our international collaborators.

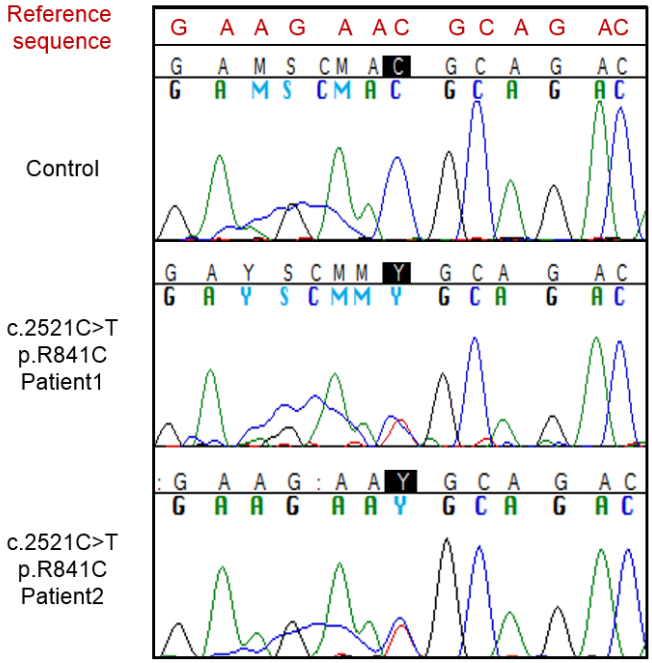


Figure 5.2 – Sequencing of p.R841C samples. Sanger sequencing of one control sample and two affected unrelated patients carrying the heterozygous *MATR3* p.R841C variant. Blue peaks represent C bases, red peaks T bases, black peaks G bases and green peaks A bases.

The remaining two variants (p.P368T and p.S748P) were either found at least once in the exome databases, predicted to be benign or to affect splicing (Table 5.3), and were not prioritised for further investigation. N-terminal HA tagged clones of mutants p.R841C, p.R317C, p.G153C, p.F115C and p.S85C were made in a Gateway pDEST-30 vector (see section 2.4.1) however, due to technical complications and multiple attempts, the p.R317C mutant could not be made and therefore was excluded from the study.

Constructs carrying two of the mutations published by Johnson and colleagues in 2014, p.S85C and p.F115C were generated, in order to investigate their pathogenic

mechanism further. We chose to study p.S85C as it was reported to have a different functional phenotype compared to WT and p.F115C which was shown to mislocalise and aggregate in post mortem tissues.

5.4.3 *MATR3* variant p.R841C is not enriched in an ALS population of >5000 patients

The *MATR3* variant p.R841C was observed three times in a cohort of 5,217 patients and 19 times in 83,079 published exomes. A Chi square test followed by Yate's correction was applied to determine whether the variant frequency was significantly different between the two populations. This analysis revealed that p.R841C is not significantly enriched in this ALS population (Chi square=1.178 with one degree of freedom; two tailed p value=0.2778).

	Carrier	Non Carrier
ALS	3	5,214
Exome database	19	83,060

Table 5.4 – 2X2 contingency table illustrating the number of ALS and control cases harbouring the p.R841C variant and those that do not.

When the same statistical test was applied to our other chosen variants, only p.R841C was significantly enriched in this ALS population compared to controls at the time. However, we chose to conduct functional studies on p.S85C, p.F115C, p.G153C and p.R841C variants as no phenotypic information is available in the exome databases and they could still be pathogenic.

Exon	Variant[a]	Predicted aa change	Affected individuals (n=5,217)	Frequency in controls (n=85986)	p Value (2017)	p Value (2014)	Reference
5	c.254C<G	p.Ser85ysC	1	0	0.566	0.1240	(Johnson *et al.*, 2014)
5	c.344T>G	p.Phe115Cys	1	2	0.4142	0.1240	(Johnson *et al.*, 2014)
5	c.457G>T	p.Gly153Cys	3	0	0.0001	0.1240	(Marangi *et al.*, 2017)
18	c.2521C>T	p.Arg841Cys	3	19	0.2778	0.0119	ALSdb

Table 5.5 – Enrichment of MATR3 variants in ALS vs control populations. Table showing the variants investigated in this study and how many times they are present in the ALS and control cohorts. The p value (2017), obtained with a Chi square test followed by Yate' correction, shows whether they are enriched in the current ALS population compared to controls. p Value (2014) shows whether they were enriched in the ALS population compared to the control population available at the beginning of this study. [a] Mutation nomenclature as recommended by the Human Genome Variation society www.hgvs.org, utilising +1 as the A of the initiator Met codon, translation start site.

5.4.4 ALS-linked MATR3 variants maintain similar expression in HEK293T

HEK293T cells were transfected with HA tagged MATR3 WT, p.R841C, p.G153C, p.F115C and p.S85C. 48 hours later cells were harvested and processed for western blotting as described in section 2.6.2. The membrane was then probed for HA tag and GAPDH, with the aim of verifying whether the expression of MATR3 was altered in any of the variants compared to wild type.

MATR3 is a 125 kDa protein, however, when expressed in HEK293T cells, the most abundant HA-labelled band was at ~40 kDa band (Figure 5.3). Both bands were absent from untransfected cells and, as the clone was from cDNA, the lower band must represent a cleaved N-terminal fragment. Quantification of the ~125 kDa and ~40 kDa bands did not show any statistically significant difference between the expression of MATR3 WT and any of the variants. However, MATR3 p.R841C construct shows higher expression of full length (125kDa) proteins compared to wild type (Figure 5.3), despite not reaching significance (p=0.1896).

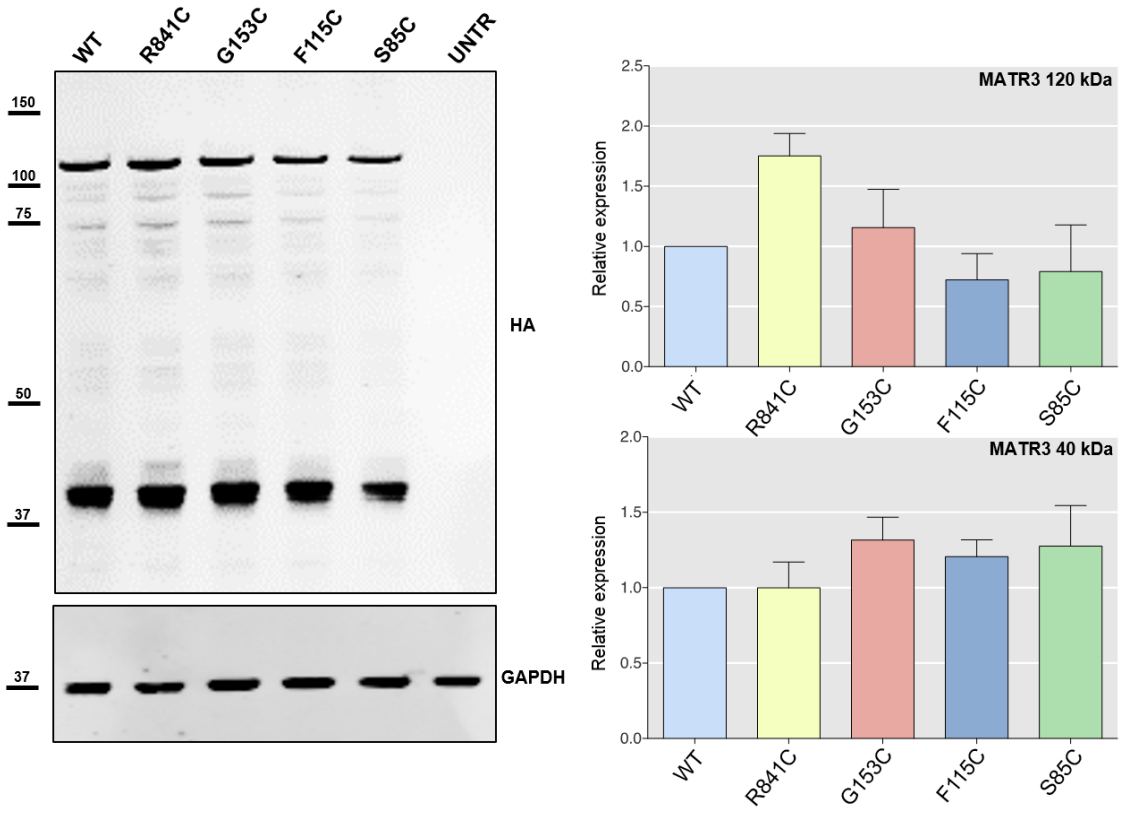
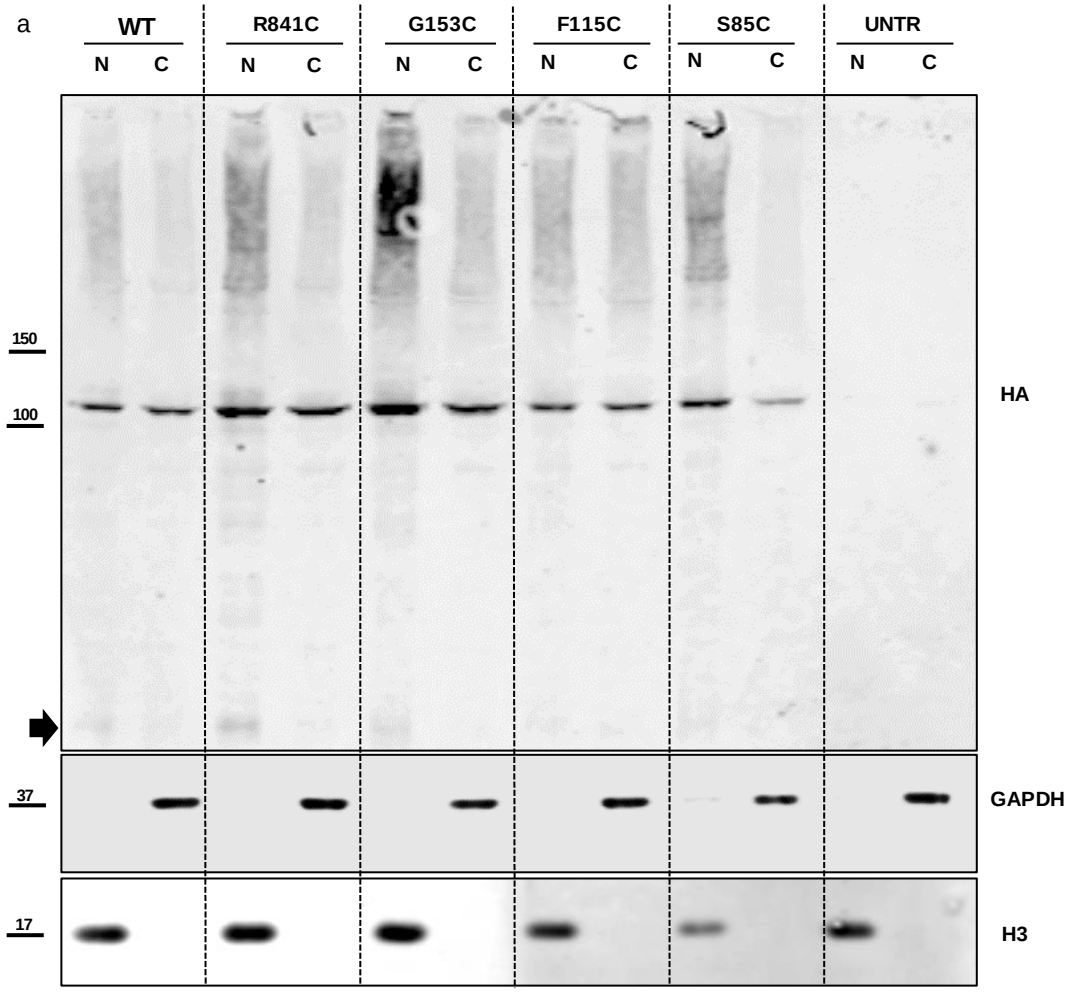


Figure 5.3 – MATR3 construct expression levels in HEK293T cells. Western blot showing MATR3 expression in WT and mutants showing the predicted ~120 kDa band as well as an additional ~40 kDa band (UNTR= untransfected). MATR3 expression was normalised against GAPDH and no statically significant difference was observed (n=3, analysed by One Way ANOVA followed by Dunnett's multiple comparison test, MATR3 120kDa p= 0.1077 and MATR3 40 kDa p= 0.5394). Error bars represent the standard error of the mean (SEM).

5.4.5 Cellular localisation remains similar among MATR3 variants

When this project was undertaken, no study had shown whether MATR3 cellular localisation was altered in the presence of possibly damaging ALS associated *MATR3* variants. Mislocalisation of the RNA binding proteins TDP43 and FUS from the nucleus to the cytoplasm of motor neurons are a key feature of ALS pathology (Neumann et al. 2006; Vance et al. 2009). To investigate whether the subcellular localisation of MATR3 was affected by mutation, a fractionation nuclear/cytoplasmic assay was performed on HEK293T cells transiently transfected with HA tagged MATR3 WT, p.R841C, p.G153C, p.F115C and p.S85C constructs (Hisada-Ishii *et al.*, 2007). No significant difference was

observed between WT and mutant MATR3 protein levels in the cytoplasmic or nuclear fraction (Figure 5.4 b and c). However, p.S85C shows a trend towards higher expression of MATR3 in the nucleus (p=0.2784) (Figure 5.4b). Further analysis revealed no significant difference in the nuclear to cytoplasmic ratio of MATR3 WT compared to any other mutant. However, a higher ratio of nuclear to cytosolic p.S85C MATR3 was observed compared WT, despite not reaching significance (p=0.1403 Figure 5.4d). Interestingly, the ~40 kDa band clearly observed in Figure 5.3 is weakly expressed only in the nuclear fraction of HEK293T transfected with MATR3 WT, p.R841C, p.G153C and p.S85C (Figure 5.4a, black arrow).



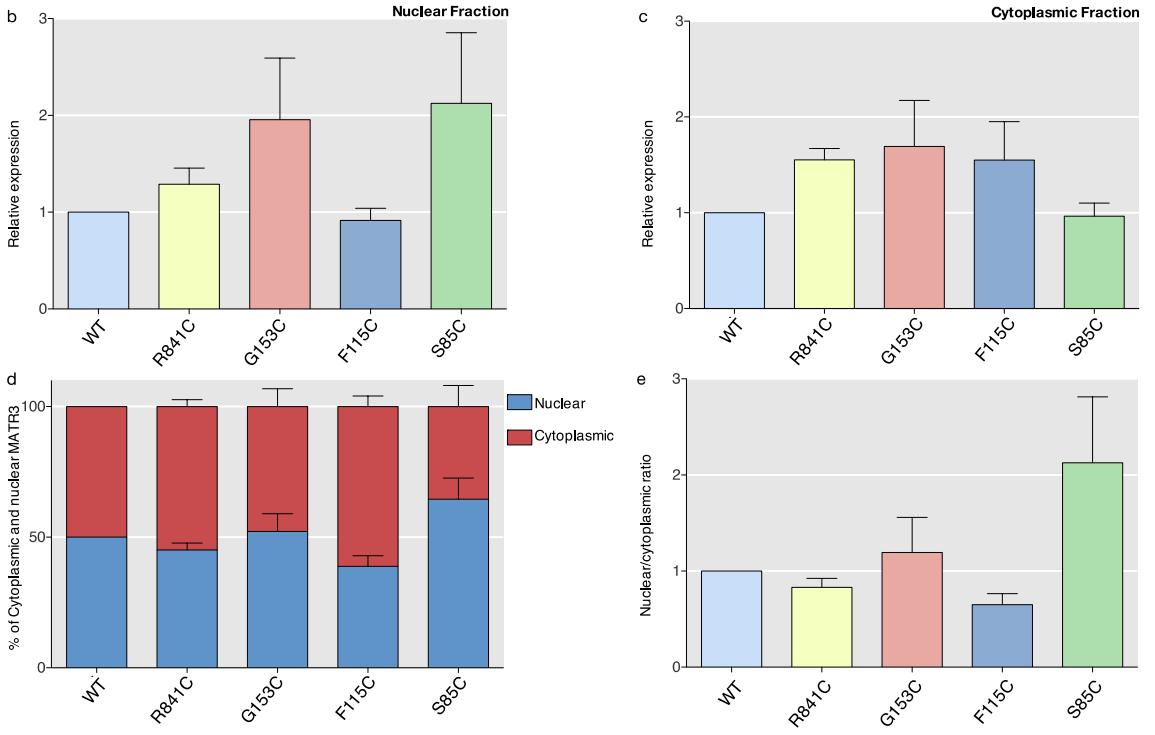


Figure 5.4 – Cellular localisation of ALS-associated MATR3 variants. (a) Representative western blot of a nuclear/cytoplasmic fractionation on HEK293T cells transiently transfected with MATR WT, p.R841C, p.G153C, p.F115C, p.S85C (black arrow pointing at the ~40 kDa cleaved MATR3) and its quantification showing the amount of nuclear (b) and cytoplasmic (c) MATR3 per sample. No significant difference was observed among samples (n=3, analysed by One Way ANOVA followed by Dunnett's multiple comparison test, (b) p=0.2586 (c) p=0.3068). (d) Graph showing the percentage of nuclear and cytoplasmic MATR3 per sample. (e) Graph showing the ratio of nuclear divided by cytoplasmic MATR3. No significant difference was observed among fractions (n=3, analysed by One Way ANOVA followed by Dunnett's multiple comparison test, p=0.0964). Error bars represent the standard error of the mean (SEM).

5.4.6 MATR3 mutants form cytosolic inclusions when expressed in HEK293T cells

We further investigated MATR3 subcellular localisation in transfected HEK293T cells using immunocytochemistry. Cells were fixed and probed with a HA tag antibody and DAPI, which revealed WT MATR3 was almost exclusively nuclear, however cytoplasmic inclusions were occasionally observed in cells expressing MATR3 p.R841C, p.G153C and p.F115C mutant constructs (Figure 5.5). Quantification revealed that all MATR3 mutants had more inclusions than WT, which was most marked for p.R841C (Figure 5.6).

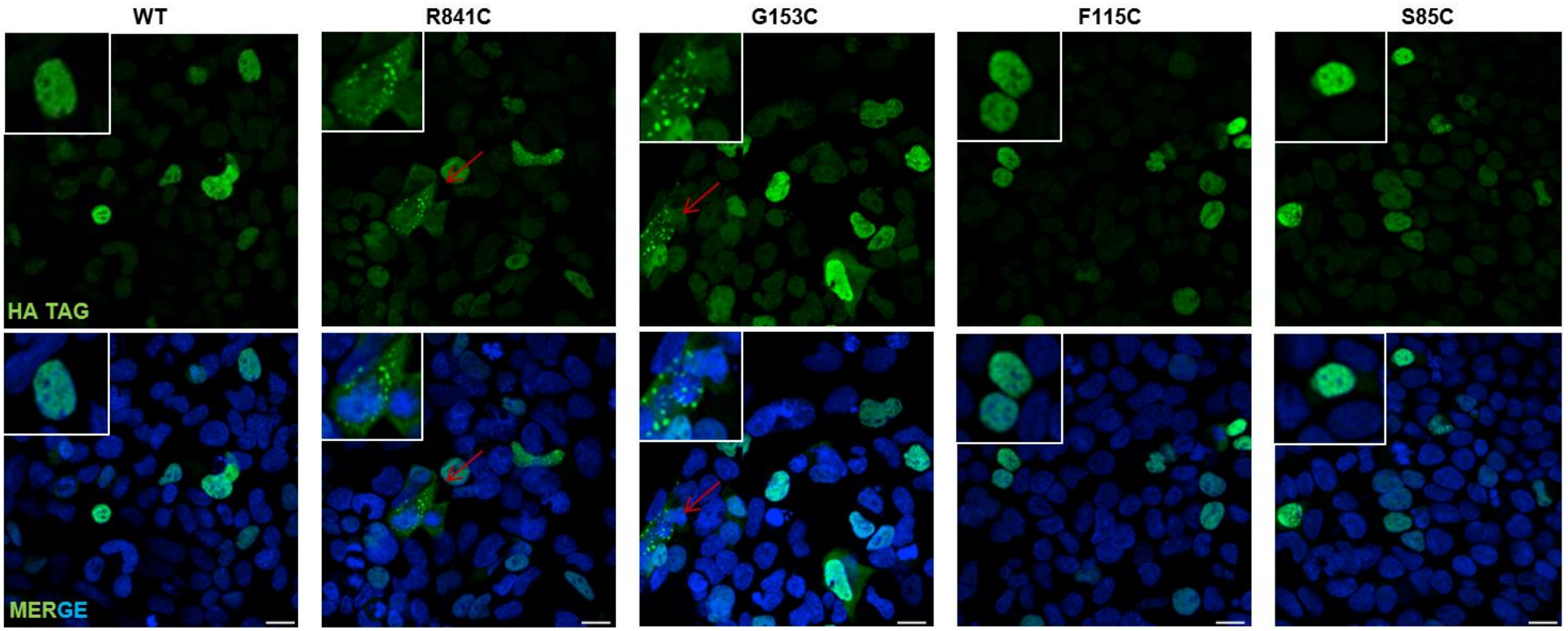


Figure 5.5 – HA staining in HEK293T cells transfected with MATR3 WT, p.R841C, p.G153C, p.F115C, p.S85C. These were probed for HA tag and DAPI staining. Transfected cells show nuclear HA TAG staining with some evidence of aggregation and cytoplasmic staining in R841C, F115 and S85C. Scale bar = 10 µM.

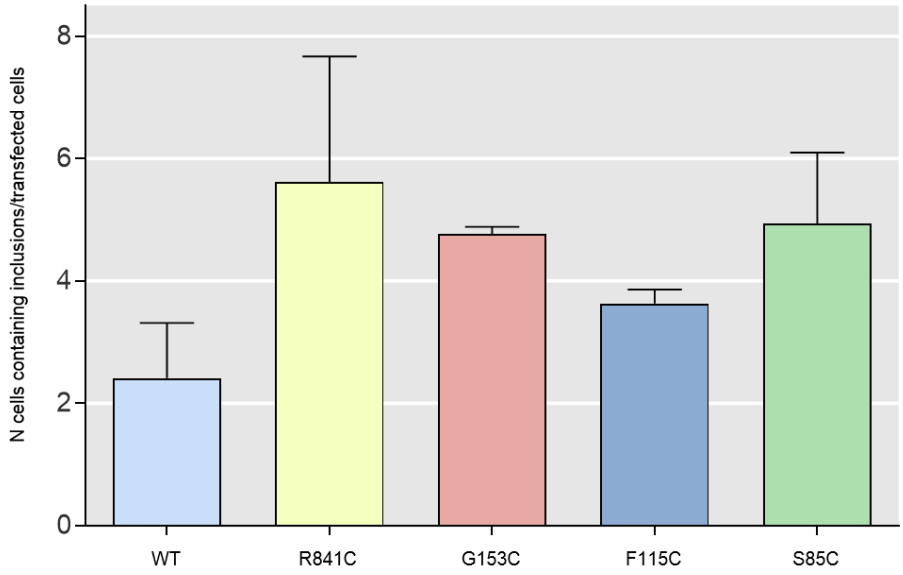


Figure 5.6 – Inclusion quantification. Quantification of the number of transfected cells containing aggregates per genotype (~600 cells counted per sample per replicate, n=2). Error bars represent the standard error of the mean (SEM).

5.4.7 MATR3 p.S85C variant shows increased insolubility in HEK293T

The presence of insoluble aggregates in cells expressing pathogenic mutations is a feature common to most neurodegenerative disorders. To determine if the aggregates visualised by immunocytochemistry (Figure 5.5) were insoluble, I conducted a complementary RIPA solubility assay with quantification analysis of MATR3 and TDP-43 in the insoluble fractions by western blot (see section 2.6.3).

Transiently transfected HEK293T cells, expressing HA tagged MATR3 WT, p.R841C, p.G153C, p.F115C and p.S85C constructs, were harvested, processed and analysed by western blotting as described in section 2.6.2. Quantification of the insoluble fraction, normalised against the amount of MATR3 present in the lysate, revealed a four-fold increase of the insoluble fraction in cells expressing p.S85C (*p = 0.0223). The amount of soluble MATR3 p.S85C was found to be reduced when compared to the wild type but was not significant (p = 0.5306).

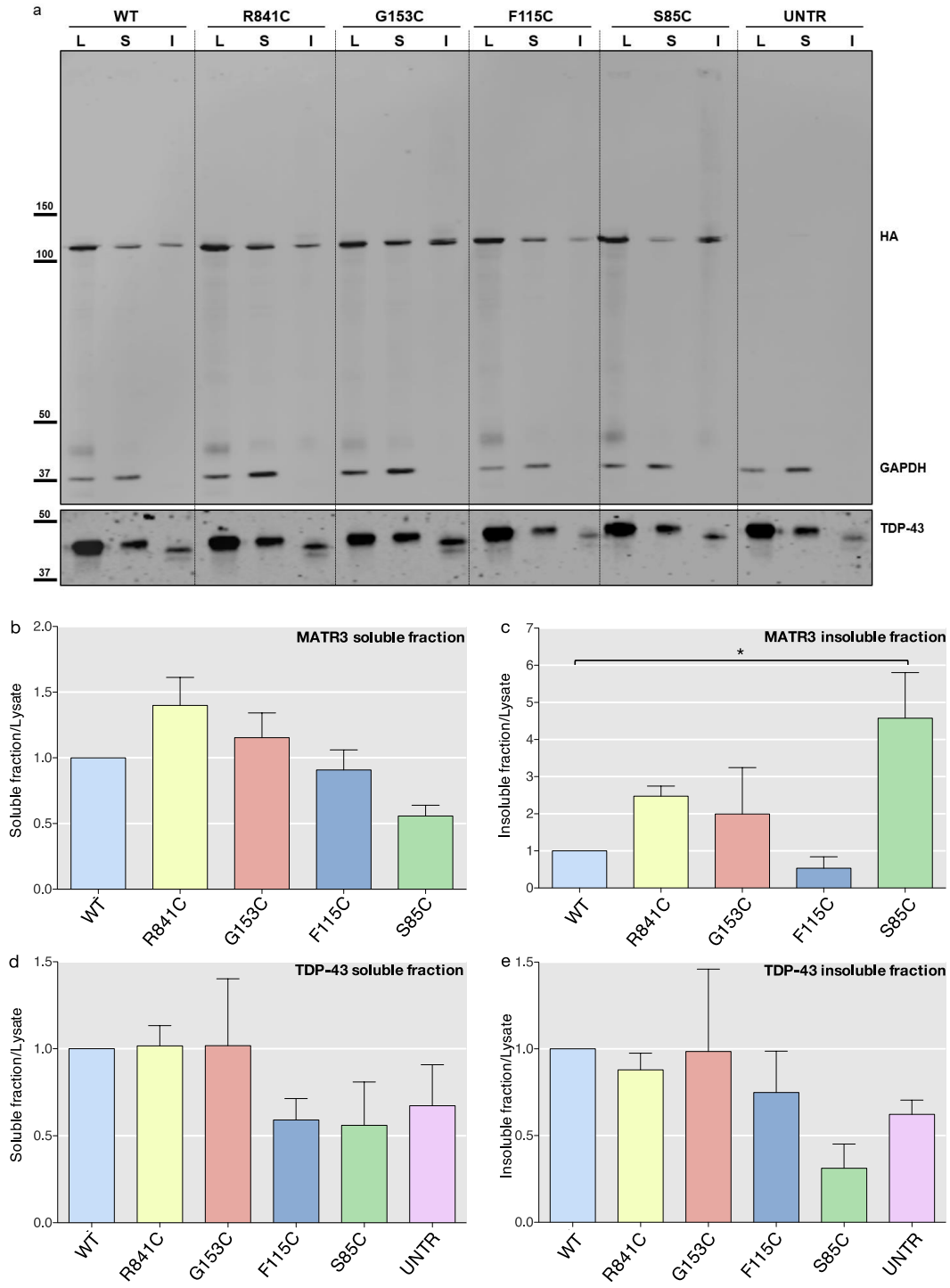


Figure 5.7 – ALS assoicated *MATR3* variant solubility analysis in HEK293T. Representative western blot of solubility assay of HEK293T cells transiently transfected with MATR3 WT, p.R841C, p.G153C, p.F115C, p.S85C (L=lysate, S=soluble fraction, I=insoluble fraction, UNTR=untransfected). (b) Quantification showing the amount of soluble MATR3 per sample normalised against MATR3 lysate (n=4, analysed by One Way ANOVA followed by Dunnett's multiple comparison test, p=0.0156). (c) Quantification showing the amount of insoluble MATR3 per sample normalised against MATR3 lysate (n=4, analysed by One Way ANOVA followed by Dunnett's multiple comparison test, *p=0.0223). (d) Quantification showing the amount of soluble endogenous TDP-43 per sample normalised against TDP-43 lysate (n=3, analysed by One Way ANOVA followed by Dunnett's multiple comparison test, p=0.4449). (e) Quantification showing the amount of

insoluble endogenous TDP-43 per sample normalised against TDP-43 lysate (n=3, analysed by One Way ANOVA followed by Dunnett's multiple comparison test, p=0.3299). Error bars represent the standard error of the mean (SEM).

It has previously been reported that MATR3 interacts with TDP-43, a protein that plays an important role in ALS pathology. Furthermore, Johnson and colleagues have also shown that MATR3 p.S85C displays a stronger interaction with TDP-43 compared to MATR3 WT (Johnson *et al.*, 2014). In the context of ALS and FTD, TDP-43 often mislocalises to the cytoplasm and creates insoluble cytoplasmic inclusions (Abeliovich *et al.*, 2012). With this in mind, we investigated the insolubility of TDP-43 in HEK293T cells transiently transfected with WT and mutant MATR3, to verify whether there was any correlation between MATR3 and TDP-43 solubility. No significant difference emerged from the comparison of mutant with WT MATR3 transfected cells. Interestingly, the amount of insoluble TDP-43 in p.S85C transfected cells is lower than the amount of insoluble TDP-43 in cells transfected with MATR3 WT, however, the comparison did not reach significance (p=0.1888, Figure 5.7e).

In order to determine whether MATR3 mutations influenced colocalisation with endogenous TDP-43, HEK293T cells were transiently transfected with HA tagged MATR3 WT, p.R841C, p.G153C, p.F115C, p.S85C and probed for HA tag (green), TDP-43 (red) and DAPI (blue). Both MATR3 and TDP-43 were predominantly localised in the nucleus showing occasional cytoplasmic aggregates as well as diffuse staining but no cytoplasmic colocalisation was observed between MATR3 and TDP-43 (Figure 5.8).

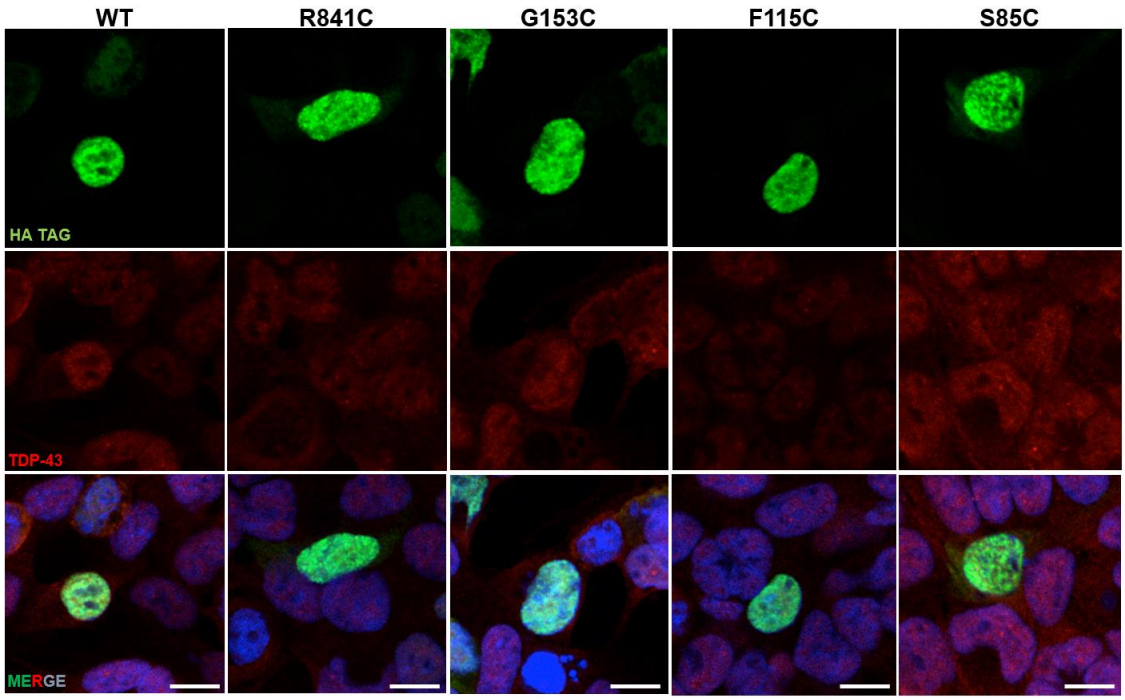


Figure 5.8 – Colocalisation of MATR3 and TDP43 in HEK293T cells transfected with HA tagged MATR3 WT, p.R841C, p.G153C, p.F115C, p.S85C. These were probed for HA tag (green), TDP-43 (red) and DAPI (blue). Transfected cells show nuclear HA tag, TDP-43 and DAPI staining. Scale bar = 10 µM

5.4.8 ALS-linked MATR3 variant p.R841C shows aggregation in SH-SY5Y cells

The neuroblastoma-derived cell line SH-SY5Y is recognised as a good model for the study of neurodegenerative mechanisms as it retains some neuronal characteristics which are not found in kidney-derived HEK293T cells (Krishna *et al.*, 2014). SH-SY5Y cells were transiently transfected with HA tagged MATR3 WT and the mutant constructs and the number of cells containing inclusions quantified (Figure 5.9Figure 5.10). p.S85C variant showed a similar amount of inclusions compared to WT, while p.R841C, p.G153C, p.F115C showed an increased number of inclusions, however, only MATR3 p.R841C reached statistical significance compared to WT (**p=0.0020,Figure 5.10), confirming what had previously observed in HEK293T cells (Figure 5.6).

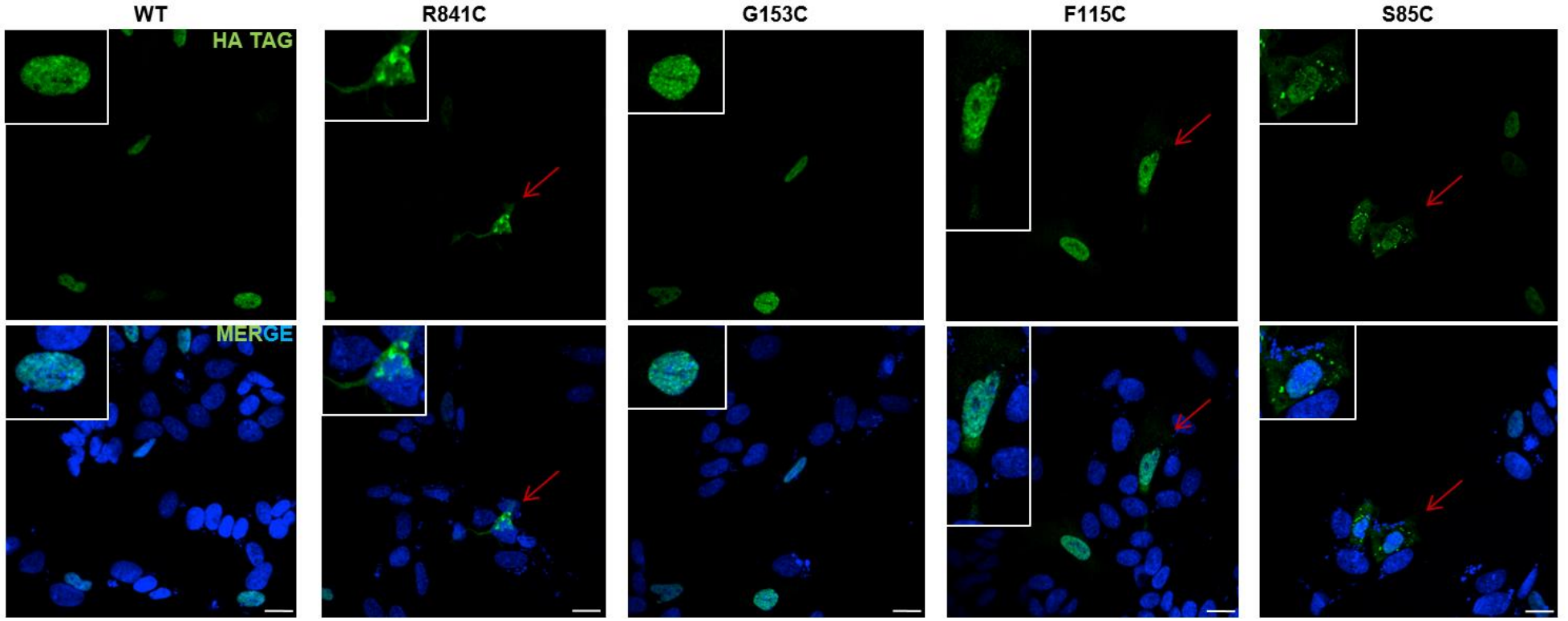


Figure 5.9 – SH-SY5Y cells transfected with MATR3 WT, p.R841C, p.G153C, p.F115C, p.S85C. These were probed for HA tag and DAPI staining. Transfected cells show nuclear HA TAG staining with some evidence of inclusions and cytoplasmic staining in R841C, F115 and S85C. Scale bar = 10 µM.

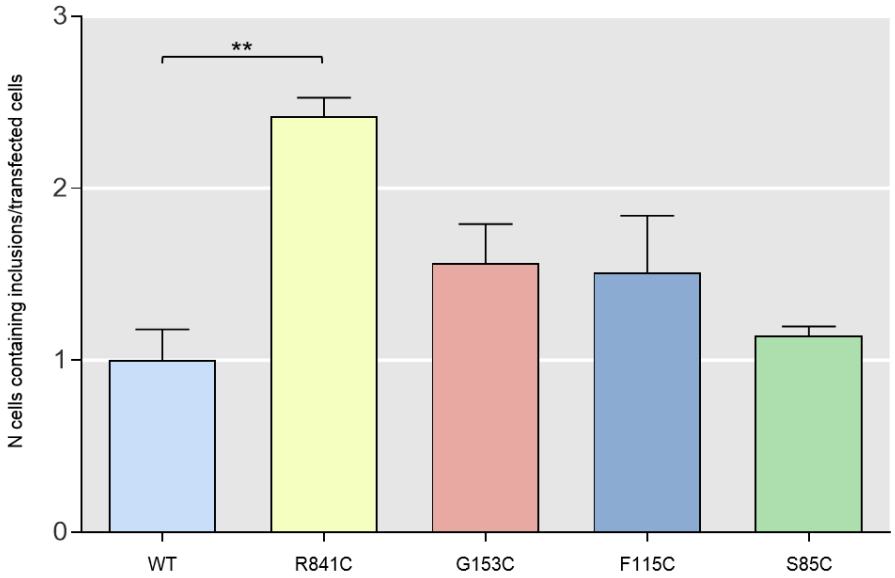


Figure 5.10 – Inclusions quantification. Quantification of the number of transfected cells containing aggregates per genotype (~250 cells counted per sample). A significant difference is observed between the number of MATR3 WT and MATR3 p.R841C transfected cells containing aggregates (n=3, analysed by One Way ANOVA followed by Dunnett's multiple comparison test, **p=0.0020). Error bars represent the standard error of the mean (SEM).

5.5 Discussion

The aim of this project was to genetically and functionally characterise ALS-associated MATR3 variants. This chapter describes five ALS-linked MATR3 variants that have been identified through exome sequencing project of 699 fALS cases. These mutations were originally filtered against ~12,600 controls and, a subset of variants (p.G153C, p.R317C and p.R841C) were chosen for functional investigation. This decision was made based on public exome databases available at the time. The variant p.R841C was initially found to be enriched in our ALS cohort in 2014 but was no longer significant in 2017 (Table 5.5), as the same variant was later found to be present a total of 19 times in 83,079 controls and 3 times in a 5,217 ALS patients. When the same analysis was applied to the other mutations none of these reached statistical significance in the ALS population, with the exception of p.G153C. It is important to note that highly powered burden analysis failed to identify confirmed ALS genes such as *PFN1*, *OPTN* and *VAPB* to as reaching

statistical significance for association with fALS and sALS (Kenna *et al.*, 2016). Moreover, the single variant chi-square enrichment test as well as burden analysis tests do not take into account the segregation of the p.S85C and p.F115C *MATR3* variants with disease in pedigrees, as illustrated by Johnson and colleagues (Johnson *et al.*, 2014), and the fact that we have found the p.R841C variant twice in our cohort of fALS. Lastly, little is known about the majority of publicly available exome controls. ALS is predominantly a late onset disease and invariably lowly penetrant (as evidenced by sALS cases), so it cannot be excluded that a proportion of these controls may develop ALS later in their life. Even in the absence of segregation or burden analysis our rare variants do merit functional studies to determine whether they might disrupt MATR3 function and be pathogenic.

From a clinical point of view, the patients belonging to our cohort do not seem to show the same characteristics (Table 5.3) as the ones reported in the literature (Table 5.1). More specifically, the patient from our cohort harbouring p.G153C variant does not show longer survival that was previously reported (Marangi *et al.*, 2017). Interestingly, mutations in other ALS genes, such as *VCP* (Johnson *et al.*, 2010) and *ATX2* (Van Damme *et al.*, 2011), have shown heterogeneous rate of survival (Li and Wu, 2016). However, a larger pool of MATR3 patients is needed in order to understand the clinical traits of MATR3-linked ALS.

When transfected in HEK293T cells, our MATR3 variants showed similar levels of protein expression (Figure 5.3), which does not confirm the report by Johnson and colleagues, who detected a lower expression of p.S85C mutant in HEK293FT cells (Johnson *et al.*, 2014). When overexpressed in HEK293T all MATR3 constructs consistently show a discrete ~40 kDa band, which is likely to be the result of a cleavage event. Since all of our MATR3 have a 3kDa N-terminal HA tag, the fragment observed represents the first ~37 kDa of the protein. Interestingly, a recent study reports that, in mice, MATR3 shows an ~80 kDa fragment that was detected using an antibody raised

against the C-terminal region of MATR3 (Rayaprolu *et al.*, 2016), so the 37kDa fragment we observed could represent its complementary N-terminal fragment. It would be interesting to verify whether, if stained with an N-terminal MATR3 antibody, the same lysates from mouse tissue also detected a ~40 kDa band. However, the abundance of these bands could be due to the overexpression of MATR3 and might not be detectable at endogenous level.

In the context of ALS, RNA binding proteins, which are predominantly confined to the nucleus, often mislocalise to the cytosol and aggregate. In our studies MATR3 remained predominantly in the nucleus and no shift to the cytoplasm was detected (Figure 5.4). If anything, the nuclear proportion of MATR3 p.S85C protein was increased although this did not reach statistical significance (Figure 5.4e). This is largely in accordance with the recent observation that the expression of MATR3 p.S85C, p.F115C, p.P154S and p.T622A in Chinese Hamster Ovary (CHO) cells did not affect the nuclear localisation of MATR3 (Gallego-Iradi *et al.*, 2015).

The formation of insoluble cytoplasmic aggregates in affected motor neurons is another common characteristic of RNA binding proteins such as TDP-43 and FUS. The solubility of MATR3 WT, p.R841C, p.G153C and p.F115C, was not significantly different from WT however p.S85C was significantly more insoluble (Figure 5.7). Johnson and colleagues have found that MATR3 p.S85C coaggregates with TPD-43 in skeletal muscle tissue (Johnson *et al.*, 2014). Surprisingly, the level of endogenous TDP-43 was relatively decreased in both the soluble and insoluble fractions of cells transfected with p.F115C and p.S85C, although this did not reach statistical significance.

We were unable to show any correlation between the solubility and aggregation of ALS-linked MATR3 variants and TDP-43 in transiently transfected HEK293T cells. It would be interesting to replicate the immunoprecipitation study reported by Johnson's and colleagues to determine whether p.R841C and p.G153C cause any impairment of the MATR3-TDP43 interaction. Furthermore, since MATR3 has been shown to interact with

TDP-43 in an RNA dependent manner (Johnson *et al.*, 2014), an informative experiment would be to check whether any of the mutations in our cohort disrupted MATR3-RNA interactions at the RRM2 domain (Salton *et al.*, 2011).

The presence of insoluble MATR3 in the nucleus could cause an impairment of MATR3's ability to cooperate with SFPQ and NONO. MATR3, in fact, interacts with these two proteins in order to perform non homologous end joining (NEHJ) and repair double strand DNA damage (Salton *et al.*, 2010). Checking for MATR3 interaction with SFPQ and NONO, by immunoprecipitation for instance, would reveal whether our subset of mutations disrupts MATR3 ability to coordinate NEHJ. In this process, MATR3 is known to be phosphorylated by ATM. Lack of MATR3 phosphorylation has been found in patients with ataxia-telangiectasia (A-T) (Salton *et al.*, 2010). Another interesting experiment would be to verify whether any of our variants causes lack of MATR3 phosphorylation, resulting in an impairment of DNA double strand break repair, which could cause neuronal toxicity.

Furthermore, the increased presence of p.S85C in the nucleus could also disturb MATR3 regulation of mRNA splicing. MATR3 acts as a splicing repressor by interacting with PTB, an RNA binding protein that regulates mRNA splicing (Coelho et al. 2015). It would be interesting to determine whether any of these variants affected MATR3's ability to bind PTB and disrupted alternative splicing. The impairment of these mechanisms could be particularly deleterious for tissues where there is a minimal cell turnover, such as neurons or muscles which bear the burden in mutant MATR3 cases. Interestingly, the *MATR3* variant p.S85C has been linked to both asymmetric myopathy (Senderek *et al.*, 2009) and ALS (Johnson *et al.*, 2014).

The biggest limitations of this study is that all the experiments have been conducted in overexpression models using cell lines that do not reflect the physiology of neuronal cells. This was due to the lack of availability of patient tissue. Although, the cell lines used in this study are widely accepted as a good model for biochemical and molecular

biology studies, it would be interesting to replicate some of these assays in human iPS-derived cells, expressing the *MATR3* variants endogenously, in order to verify whether the same dysfunction is observed.

5.6 Conclusions

In summary, we have shown that the MATR3 variant p.S85C is relatively insoluble compared to MART3 WT and that it has a tendency to be retained in the nucleus. Furthermore, the MATR3 variant p.R841C forms cytoplasmic inclusions but these were not insoluble. These two mutations could disrupt the low complexity domain of MATR3 and, in a similar way to TDP-43 mutations, they could increase its solubility and tendency to form inclusions (Coelho et al. 2015), however the MATR3 variants did not increase TDP-43 insolubility and MATR3 and TDP43 did not colocalise within inclusions.

The behaviour of MATR3 p.F115C and p.G153C variants was no different from WT in this overexpression model and we have no evidence for their pathogenicity. As we do not know the mechanism of action for MATR3 mutations it may be that these mutations are pathogenic by a by different molecular mechanisms and further investigation is needed in order to clarify the role of MATR3 mutations in ALS pathogenesis.

Chapter 6 - General Discussion

The general aim of this thesis was to shed light on ALS pathogenesis by studying the molecular mechanisms involved in newly identified genes: *MATR3*, *OPTN* and *TBK1*. When this project was initiated, my team and their international collaborators, had just completed an exome sequencing project of ~700 index familial ALS patients, with the aim of identifying novel genes casually implicated in ALS. This project has successfully identified novel ALS-associated genes, including *TUBA4A*, *ANXA11* and *NEK1* (Smith *et al.*, 2014, 2017; Kenna *et al.*, 2016). Another important contribution from exome sequencing is the identification of more mutations in recently discovered genes, which presents an opportunity to study their functional effects and map mechanistic pathways. We believe that studying the pathogenic mechanisms for these mutant genes will provide further insights that may contribute towards finding novel therapies.

Through our exome sequencing, we identified sixteen *TBK1* variants four of which were novel. In the first result chapter of this thesis I show that two ALS associated *TBK1* variants, the missense p.G217R and the truncation p.R357X mutations both showed a significant loss of TBK1 kinase activity while another variant, p.C471Y, did not. Many ALS-linked nonsense mutations were implicated in haploinsufficiency as a primary disease mechanism, however relatively little focus has been directed at understanding how missense mutations might cause disease (Freischmidt *et al.*, 2015; Gijselinck *et al.*, 2015; Kim *et al.*, 2016; Tsai *et al.*, 2016; Pozzi *et al.*, 2017). The loss of TBK1's ability to auto-phosphorylate has only been observed once before in an overexpression model of in frame deletions (van der Zee *et al.*, 2017) and never in missense variants or patient material. In this study I demonstrated that patient derived lymphoblast harbouring *TBK1* missense variants show a lower level of TBK1 phosphorylation, regardless of where the variants map in the functional domains. Furthermore, I also showed that the missense mutation p.G217R, which lies in the kinase domain, disrupts the ability of TBK1 to homodimerise and auto-phosphorylate and, therefore, to carry out its physiological

function, suggesting a common mechanism for all missense and nonsense mutations. This would lead to a TBK1 haploinsufficiency causing a functional impairment of autophagy and inflammatory response, both of which have previously been implicated in ALS and FTD (Cirulli *et al.*, 2015; Freischmidt *et al.*, 2015). I demonstrated that these two TBK1 mutants failed to phosphorylate IRF3, as had previously been observed (Freischmidt *et al.*, 2015; Kim *et al.*, 2016; Tsai *et al.*, 2016; Pozzi *et al.*, 2017). This in turn inhibits NF-kB/IFN activation and suggests an involvement of the inflammatory pathway (Freischmidt *et al.*, 2015; van der Zee *et al.*, 2017). However, some variants retain the ability to regulate this pathway and therefore this is not a mechanism common to all *TBK1* mutations. Post-mortem brain analysis of ALS and FTD patients bearing mutations in *TBK1* have shown accumulation of TDP-43 aggregates (Gijselinck *et al.*, 2015; Pottier *et al.*, 2015; Koriath *et al.*, 2016; van der Zee *et al.*, 2017), which implicates a disruption of autophagic clearance. Furthermore, ALS-associated nonsense variants, or those mapping in the scaffold dimerization domain (SDD) (p.L59Ffs, p.R444X, p.Ile472Serfs*8, p.M559R, p.E696K, p.690-713del) have been shown to affect TBK1 binding with OPTN (Freischmidt et al. 2015; Kim et al. 2016; Pozzi et al. 2017; Richter et al. 2016; Tsai et al. 2016). I have shown that the mutation p.G217R, which maps in TBK1's kinase domain (KD) also affects TBK1 binding to OPTN. Therefore, autophagy impairment remains a valuable hypothesis. However, the involvement of TBK1 in so many pathways makes it hard to point the finger at one singular mechanism, and it may well be the case that TBK1 associated pathology results from an ensemble of different defective cellular pathways.

Exome sequencing and shared region of homozygosity analysis identified a novel nonsense *OPTN* mutation (p.S174X) in a consanguineous Palestinian family presenting with an aggressive adult-onset form of ALS in four siblings. Through our collaborators I obtained fibroblasts derived from four asymptomatic family members (three heterozygous and one homozygous for the truncation mutation) and demonstrated that

when harbouring the mutation p.S174X OPTN mRNA is degraded by nonsense mediated decay (NMD), resulting in a complete or partial loss of OPTN expression in homozygous and heterozygous carriers respectively. The absence of OPTN expression in homozygous individuals is likely to be responsible for their disease. Because OPTN has been involved in the mitophagy pathway together with TBK1 (Ying and Yue, 2016), I explored this further in the fibroblast lines. Mild mitochondria respiratory dysfunction was detected in the heterozygous lines but curiously not in the homozygous line derived from an unaffected sibling. It may be that OPTN null cells develop a compensatory mechanism that may fail with aging or that this individual has another protective mechanism absent from her siblings. Analysis of more homozygous cell lines might provide an explanation. Accumulation of the autophagy markers LC3-II and K-63 ubiquitin was not observed in any of the fibroblast lines and no striking difference was observed in the number of TDP-43 inclusions and their colocalisation with p62 among fibroblast lines. Primary fibroblasts, however, have a slow metabolism and it is possible that the heterozygous and homozygous fibroblast lines would show defects in autophagy if stressed using autophagy inhibitors or other cellular stressors.

The exome sequencing project also identified five *MATR3* variants that, at the time, had not been described before, and one of which (p.R841C) was found in two unrelated patients. I carried out overexpression studies in HEK293T cells on two of these variants (p.G153C and p.R841C) together with two of the variants (p.S85C and p.F115C) first identified by Johnson and colleagues (Johnson *et al.*, 2014). I have demonstrated that the p.S85C MATR3 mutant is more insoluble when compared to the wild type, or other mutants. Mutant protein insolubility and inclusion formation are characteristics shared by other RNA binding proteins linked to ALS, including TDP-43 and FUS (Kwiatkowski *et al.*, 2009; Scotter *et al.*, 2014). I have also shown that the MATR3 mutant p.R841C enhances aggregate formation in two cell line models, reaching significance in SH-SY5Y cells, a neuroblastoma-derived cell line that preserves some neuronal characteristics

(Kovalevich and Langford, 2013). Aggregate accumulation of RNA-binding proteins, as well as other proteins, is also a feature observed in the brain and spinal cord of ALS and FTD patients (Peters, Ghasemi and Brown, 2015). Therefore, both these findings are interesting and could point towards a possible MATR3-disease causing mechanism.

I believe that, in this thesis, I have highlighted the importance of follow up functional studies, starting from an anonymous investigation technique such as WES. Functional characterisation is necessary in order to confirm the pathogenicity of a variant, even if this is predicted to be pathogenic. As I observe in the case of *TBK1* variant p.C471Y, predicted to be pathogenic by 20 algorithms, this does not show any sign of pathogenicity according to the assays used in this study. This might mean that the variant is not pathogenic at all. Without the functional characterisation we would have not been able to come to such conclusion. It is possible that the p.C471Y variant may cause the disease through a different pathway, however a result similar to what we observe for p.C471Y, was obtained by Kim and colleagues for the variant p.I475T (Kim et al. 2016). This might indicate that *TBK1* variants that map in that particular region of the protein do not have a major impact on TBK1 function. A similar case is observed in MATR3, where the variant p.F115C and p.G153C are predicted to be pathogenic but do not show evidence of pathogenicity in our as well as other published studies (Gallego-Iradi *et al.*, 2015; Marangi *et al.*, 2017; Iradi *et al.*, 2018). Overall, I believe that this thesis emphasises the importance of functional variant characterisation as an essential tool to demonstrate a variant pathogenicity in the context of ALS.

Many of the studies presented here employ overexpression, which are robust and widely used models to study neurodegenerative diseases but they also represent a limitation. Indeed, immortalised cell lines are known to collect genetic abnormalities when cultured for an extended period of time. Furthermore, the ability of these cells to replicate continuously and the high level of protein expression induced by the transfection do not accurately represent physiological or disease conditions in the brain. These models are

relatively quick and easy to manage and have played an important role in the discovery of many underlying mechanisms of neurodegeneration. For these reasons, we decided to adopt this method as an initial investigation of how the novel mutations found by exome sequencing would influence cell homeostasis.

A similar argument can be applied to my use of non-neuronal patient derived cell lines. Although they carry the patient genome and express the mutated proteins endogenously, they do not necessarily recapitulate the neuronal pathology. However, research using these cells is still valuable as we assume that dramatic changes observed in fibroblast or lymphoblast cell lines, such as the absence of OPTN or the lack of TBK1 phosphorylation, may also be present in neurons. Furthermore, these cells can be reprogrammed to become induced pluripotent stem cells (iPSCs), which can then be differentiated into motor neurons to generate more physiological model for the study of this disease.

An additional limitation of this thesis is the amount of available lines carrying the same ALS-associated variant. Throughout this study we often had access to only one line carrying each *TBK1* or *OPTN* mutation (with the exception of the fibroblast lines carrying the homozygous p.S174X *OPTN* change). This is due to lack of available patients carrying the same mutations. A solution to this problem would be to artificially introduce the variant in iPSCs, using the CRISPR-Cas9 system (Sander and Joung, 2014) for instance, that can successively be differentiated into motor neurons. This would be an ideal approach as control and mutated lines would be genetically identical, making any phenotypic change attributable to the introduced mutation.

In conclusion, from exome sequencing I have identified several novel mutations in three genes recently associated with ALS: *TBK1*, *OPTN* and *MATR3* and explored their functional consequences in simple cellular model to generate new insight into disease pathogenesis. While acknowledging the limitations of an overexpression system I believe

that several findings in this thesis have made a modest contribution to our knowledge of the molecular mechanisms driving neurodegeneration in ALS. It is only through a greater understanding of fundamental disease mechanisms that we will be able to identify potential therapeutic targets and develop more effective therapies for this dreadful disease.

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Appendix A. Primer List

6.1 Primers used for genotyping of *TBK1*, *OPTN* and *MATR3*

Gene	Exon	Forward primer	Reverse primer
TBK1	2	CATGTCAGTAACAGATAATGGGTGA	CAAAGGGATCAAAATCACCAA
	3	TCCCTGTGCCTAAAAGATGC	TGAAAATTAGGGTTTGTACAACCA
	5	AATGAATTTGAGACATGCACACA	AGGGTGCTACAAAACTTCCAAG
	6	AAAGGGCATAAGGGAGTGCT	TGGGAGGAAAGTGCTGACAA
	7	TGGATCCTGTGAGCATCAATC	TTGAGTATCTGTGGTTCTAGGCA
	8	CAGTTCCTTTGATTTGCTGGT	TGGGAAATGGGTACTTTTGG
	9	TGCCTCTCACTTTATCCCCA	ACACAATGAGTCCAATGCTTCT
	12	TACATCAGGATCACAGAAATGCT	AGCAGGCCTCAAGTCTTAGT
	18	TGCCACAAAAGCTATGACGC	GGGTGGAACTGAATGTAGGC
OPTN	6	GGTGGCCAGCCTTAGTTG	TTGTTTCATCTTTCCAGGGG
MATR3	5	ACGGCATCTGCTTAAAGGGA	TGCTTGATCAACTCAATGTCATCA
	6	ATGGGCAGGTGTTTGTACAG	TGTCCATACAGTACCACTATGTGA
	7	AGGCTGTTTTGTGAAAAGGACA	TGTTGAAAGTGAAACCTGAATTCCT
	8	TGTGGTCATATATTGGGGTGCT	GGGCGATAAGAGCAAAACCC
	9	TGTGTTTGGAAGATGCACGT	AGGACCACGATACATACTCAACT
	10	AGTCCTAATGCGTAATTGGTTTCA	CGAGACTCCGACTCCCAAAA
	12	TTGCACATTTGTCCTTTTGTTGT	GAATGACGCCCAGCTCCATA
	14	CCCTAAAGGACACCTTCATTGC	TGGAGATTCTTTGCCATCTGGA
	16	CAGACAGAAGGGATGCTGCA	AGGAGAGTGAGGAAGGTGAAGA
	17	AGGCTGTATTGGACTTCTCAAA	TCAGGAGAAAGCACTGCACT
	18	TGCTGCATTTCTCTTAGGTGAC	CCAAAACGGGTTCAAACTCCA

Table 6.1 – Sequence of all the primers used for genotyping of *TBK1*, *OPTN* and *MATR3*. Forward and reverse primers were used for the amplification and sequencing of the different exons. Primers were designed using Primer 3 as described in section 2.3.1. Sequences are represented 5' – 3'.

6.2 Mutagenesis primers

Gene	Variant	Forward primer	Reverse primer
TBK1	p.G217R	GGTCTAAATGGCAGTGATCTAGTAGCTGCATGGTAAAAT	ATTTTACCATGCAGCTACTAGATCACTGCCATTTAGACC
	p.R357X	GTTCTAAGACTAAGCGTCACCCTTCGTAGATAAGTTC	GAACTTATCTACGAAGGGTGACGCTTAGTCTTAGAAC
	p.C471Y	CACAGTTTTTTCAATGTTTCTGATATAGAAATCCAATGTGATCACAACTTC	GAAGTTGTGATCACATTGGATTTCTATATCAGAAACATTGAAAAAACTGTG
MATR3	p.S85C	CTCTACTTCCAATGTTAAATATACACTGCAAATTATGGGAAGAAGTA	TACTTCTTCCCATAATTTGCAGTGTATATTTAACATTGGAAGTAGAG
	p.F115C	GTAACATTTTGGCCAGCTGTGGTCTGTCTGCTAG	CTAGCAGACAGACCACAGCTGGCCAAAATGTTAC
	p.G153C	GCTTAAAAGGAGGAGAACTGAAGAATGCCCTACCTTGAG	CTCAAGGTAGGGCATTCTTCAGTTCTCCTCCTTTTAAGC
	p.R841C	CTGAATAAATTGGCAGAAGAATGCAGACAGAAGAAGGAAAC	GACTTATTTAACCGTCTTCTTACGTCTGTCTTCTTCCTTTG

Table 6.2 – Primers used for mutagenesis. Primers were designed using the Agilent QuickChange® Primer Design Program. Sequences are represented 5’ – 3’.

6.3 Primers used for cDNA sequencing

Gene	Orientation	Sequence
TBK1	Reverse	TCTTGTTGTTGTCTCCTCTTCAA
	Reverse	GCCATACAGAGAAACAAACTGCT
	Reverse	GCATGTCTCCACTCCAGTCA
	Reverse	CGTCGCCCTTCGTAGATAAGT
	Reverse	TCAGCCATCGTATCCCCTTT
	Reverse	TTTCGGATGAGTGCCTTCTT
	Forward	TGACCCCAATTTATCCAAGTT
	Reverse	CTAAAGACAGTCAACGTTGCGAAG
MATR3	Reverse	ACCCTGGTTCATCCTTCCAAG
	Reverse	GTATGGTGGCTCCCGTGTAG
	Reverse	TGTTGAAAGTGAAACCTGAATTCCT
	Forward	TGACCATGGAAGTCGTTCTCA
	Forward	ACATCATGGATTTTCAACGAGGG
	Forward	ACTGATGGTTCCCAGAAGACTG
	Forward	ACGAAGCAGCGTTGGAAAAT
	Forward	ATTGCAGCAGCCTTCCTCAT

Table 6.3 – Primers used for the sequencing of *TBK1* and *MATR3* plasmids. Primers were designed using Primer 3 as described in section 2.3.1. Sequences are represented 5' – 3'.

Appendix B. TBK1 variants discovered to date.

Type of variant	Exon	Variant[a]	Predicted aa change	Affected individuals	Affected domain	Frequency in ExAC	Diagnosis	Reference
Nonsense	2	c.4C>T	p. Gln2X	1	-	0	FTD	(Gijselinck *et al.*, 2015)
	4	c.349C>T	p.Arg117X	3	KD	1	2FTD,ALS	(Cirulli et al. 2015; Pottier et al. 2015; van der Zee et al. 2016)
	5	c.379C>T	p. Arg127X	1	KD	0	ALS	(van der Zee *et al.*, 2017)
	6	c.555T>A	p. Tyr185X	2*	KD	0	2fALS	(Freischmidt *et al.*, 2015)
	8	c.1069C>T	p.Arg357X	2	ULD	0	2ALS	(Cirulli et al. 2015)
	11	c.1340+1G>A	p.Ala417X	5	SDD	0	fALS,3sALS,FTD	(Freischmidt *et al.*, 2015; van der Zee *et al.*, 2017)
	11	c.1318C>T	p.Arg440X	3*	SDD	0	fALS,ALS-FTD,ALS	(Freischmidt *et al.*, 2015)
	11	c.1330C>T	p.Arg444X	1	SDD	1	ALS-FTD	(Tsai *et al.*, 2016)
	11	c.1331G>A	p.Arg444X	1	SDD	0	ALS-FTD	(Borghero et al. 2015; Cirulli, et al. 2015)
	11	c.1335G>A	p.Trp445X	1	SDD	0	FTD	(van der Zee *et al.*, 2017)
	13	c.1146T>G	p.Tyr482X	1	SDD	0	ALS-FTD	(Le Ber *et al.*, 2015)
	13	-	p.Ser499X	1	SDD	0	ALS	(Cirulli et al. 2015)
	19	c.1963C>T	p. Gln655X	1	SDD	0	ALS-FTD	(Le Ber *et al.*, 2015)
Frameshift deletions	2	c.86delA	p.Lys29ArgfsX15	1	KD	0	FTD	(van der Zee *et al.*, 2017)
	3	c.176dupT	p.Leu59PhefsX16	1	KD	0	sALS	(Pozzi *et al.*, 2017)

	4	c.358+2T>C	p.Thr77TrpfsX4	1	KD	0	fALS	(Freischmidt *et al.*, 2015)
	4	c.288delT	p. Val97PhefsX2	1	KD	1	ALS	(van der Zee *et al.*, 2017)
	4	c.358+5G>A	-	1	-	-	sALS	(Pozzi *et al.*, 2017)
	5	c.467_468delCA	p. Thr156ArgfsX6	1	KD	0	ALS-FTD	(Le Ber *et al.*, 2015)
	8	-	p.T278fs	1	KD	0	ALS	(Cirulli et al. 2015)
	8	c. c.958delA	p. Thr320GlnfsX40	1	ULD	1	fALS	(Freischmidt *et al.*, 2015)
	10	c.1191delT	p. Ser398ProfsX11	1	-	0	ALS	(Gijselinck *et al.*, 2015)
	10	c.1197delC	p.Leu399fs	1	-	0	fALS	(Williams *et al.*, 2015)
	11	-	p.Val421fs	1	SDD	0	ALS	(Cirulli et al. 2015)
	12	c.1343_1346delAATT	p.Ile450LysfsX15	3*	SDD	0	3fALS	(Freischmidt *et al.*, 2015)
	12	c.1385_1388delCAGA	p.Thr462LysfsX3	2	SDD	0	2ALS	(Cirulli et al. 2015; van der Zee et al. 2017)
	12	c.1414delA	p.Ile472Serfs*8	1	SDD	0	ALS	(Kim *et al.*, 2016)
	12	c.1434_1435delTG	p.Val479GlufsX4	1	SDD	0	fALS	(Freischmidt *et al.*, 2015)
	13	-	p.Asp500fs	1	SDD	0	ALS	(Cirulli et al. 2015)
	14	c.1551_1552insTT	p.Ser518LeufsX32	1	SDD	0	ALS	(Gijselinck *et al.*, 2015)
	15	-	p.Glu550fs	1	SDD	0	ALS	(Cirulli et al. 2015)
	18	-	p.Gln629fs	1	SDD	0	ALS	(Cirulli et al. 2015)
	20	c.2070delG	p.Met690fs	1	SDD	0	ALS	(Borghero *et al.*, 2015)
In frame deletions	3	c.228+1G>A	p. Lys30_Glu76del	1	KD	0	-	(van der Zee *et al.*, 2017)
	4	c.235_237delACA	p. Thr79del	1	KD	0	ALS-FTD	(van der Zee *et al.*, 2017)

	5	c.499_501delGAT	p. Asp167del	1	KD	0	ALS	(Gijselinck *et al.*, 2015)
	8	c.992+1G>T	p. Gly272_Thr331del	1	KD-ULD	0	FTD	(Gijselinck *et al.*, 2015)
	8	c.992+4_992+7delAGTA	p. Gly272_Thr331del	1	KD-ULD	0	FTD	(van der Zee *et al.*, 2017)
	-	c.1644-5_1644-2delAATA	p.(?)	1	-	-	sALS	(Pozzi *et al.*, 2017)
	18	c.1927_1929delGAA	p.Glu643del	6	SDD	0	ALS-FTD,3FTD,2ALS	(Freischmidt *et al.*, 2015; Gijselinck *et al.*, 2015)
	18	c.1928_1930delAAG	p.Glu643del	11	SDD	0	2sALS,5dementia,1ALS,2FTD1ALS-FTD	(Freischmidt *et al.*, 2015; Gijselinck *et al.*, 2015)
	19	c.1960-2A>G	IVS18-2A>G	1	-	-	ALS-FTD	(Le Ber *et al.*, 2015)
	20	c.2138+2T>c	p.690-713del	3*	SDD	0	2fALS,1-	(Freischmidt *et al.*, 2015)
Functional missense variants	2	c.10A>G	p.Thr4Ala	1	-	0	FTD	(Le Ber *et al.*, 2015)
	2	c.77G>A	p. Gly26Glu	1	KD	0	ALS	(Le Ber *et al.*, 2015)
	3	c.140G>A	p.Arg47His	1	KD	0	fALS	(Freischmidt *et al.*, 2015)
	3	c.185T>C	p. Leu62Pro	1	KD	0	sALS	(Shu *et al.*, 2016)
	3	c.217A>G	p.Ile73Val	1	KD	5	-	(Gijselinck *et al.*, 2015)
	4	c.281T>C	p.Leu94Ser	1	KD	0	ALS	(van der Zee *et al.*, 2017)
	4	c.314A>G	p.Tyr105Cys	1	KD	0	sALS	(Freischmidt *et al.*, 2015)
	4	c.352G>A	p.Asp118Asn	1	KD	1	sALS	(Pozzi *et al.*, 2017)
	5	c.362G>A	p.Gly121Asp	1	KD	0	ALS	(Freischmidt *et al.*, 2015)
	5	c.427C>T	p.Arg143Cys	1	KD	0	ALS	(Le Ber *et al.*, 2015)

	6	c.687G>T	p.Arg229Ser	1	KD	0	ALS	(van der Zee *et al.*, 2017)
	7	c.731G>T	p. Gly244Val	1	KD	0	ALS-FTD	(van der Zee *et al.*, 2017)
	7	c.737T>C	p. Ile246Thr	1	KD	0	ALS	(van der Zee *et al.*, 2017)
	8	c.871A>G	p.Lys291Glu	1	KD	21	FTD	(Gijselinck *et al.*, 2015)
	8	c.914T>C	p.Ile305Thr	1	KD	5	sALS	(Freischmidt *et al.*, 2015)
	8	c.916C>A	p.Leu306Ile	1	KD	36	ALS-FTD	(Pottier *et al.*, 2015)
	8	c.923G>A	p.Arg308Gln	1	ULD	-	sALS	(Freischmidt *et al.*, 2015)
	8	c.959C>T	p.Thr320Ile	1	ULD	-	ALS	(Le Ber *et al.*, 2015)
	9	c.1001T>C	p.Ile334Thr	1	ULD	2	sALS	(Shu *et al.*, 2016)
	9	c.1070G>A	p.Arg357Gln	2	ULD	1	fALS,sALS	(Freischmidt *et al.*, 2015; Pozzi *et al.*, 2017)
	10	c.1201A>G	p. Lys401Glu	1	SDD	1	FTD-AD	(Le Ber *et al.*, 2015)
	11	c.1252A>G	p.Ile418Val	1	SDD	1	FTD	(van der Zee *et al.*, 2017)
	15	c.1676T>G	p.Met559Arg	1	SDD	0	fALS	(Freischmidt *et al.*, 2015)
	15	c.1712C>T	p.Ala571Val	1	SDD	2	sALS	(Freischmidt *et al.*, 2015)
	17	c.1792A>G	p.Met598Val	1	SDD	0	sALS	(Freischmidt *et al.*, 2015)
	19	c.1985T>C	p.Met662Thr	1	-	0	ALS-FTD	(Le Ber *et al.*, 2015)
	20	c.2086G>A	p.Glu696Lys	3	CTD	2	fALS,sALS,FTD-AD	(Freischmidt *et al.*, 2015; Pottier *et al.*, 2015)
Missense variants of not	2	-	Leu11Ser	1	-	0	ALS	(Cirulli et al. 2015)

defined pathogenicity	2	-	Asn22His	1	KD	0	ALS	(Cirulli et al. 2015)
	2	-	Asn22Asp	1	KD	0	ALS	(Cirulli et al. 2015)
	2	-	Arg25His	1	KD	0	ALS	(Cirulli et al. 2015)
	3	c.127A>G	p.Ile43Va	1	KD	0	sALS	(Tsai *et al.*, 2016)
	3	c.188A>G	p.Asn63Ser	1	KD	0	-	(van der Zee *et al.*, 2017)
	5	-	p.Asn129Asp	1	KD	0	ALS	(Cirulli et al. 2015)
	5	-	p.Val132Glu	1	KD	0	ALS	(Cirulli et al. 2015)
	5	-	p.Arg134His	1	KD	0	ALS	(Cirulli et al. 2015)
	5	-	p.Ser151Cys	1	KD	0	ALS	(Cirulli et al. 2015)
	5	-	p.Ser151Phe	1	KD	1	ALS	(Cirulli et al. 2015)
	6	c.550A>G	p.Met184Val	1	KD	2	-	(van der Zee *et al.*, 2017)
	6	-	p.Gly217Arg	1	KD	1	ALS	(Cirulli et al. 2015)
	6	-	Arg228His	1	KD	1	ALS	(Cirulli et al. 2015)
	7	-	Ile257Thr	1	KD	0	ALS	(Cirulli et al. 2015)
	7	c.794T>C	p.Val265Ala	1	KD	0	-	(van der Zee *et al.*, 2017)
	7	c.812G>T	p.Arg271Leu	1	KD	0	FTD	(Gijselinck *et al.*, 2015)
	8	-	p.Leu277Val	1	KD	0	ALS	(Cirulli et al. 2015)
	8	c.871A>G	p.Lys291Glu	1	KD	21	sALS	(Pozzi *et al.*, 2017)
	8	c.881G>A	p.Gly294Asp	1	KD	0	sALS	(Tsai *et al.*, 2016)
	8	c.964C>T	p.His322Tyr	1	ULD	62	sALS	(Gijselinck *et al.*, 2015)

	8	-	p.Thr331Ile	1	ULD	3	ALS	(Cirulli et al. 2015)
	9	-	p.Thr343Ser	1	ULD	2	ALS	(Cirulli et al. 2015)
	9	c.1057A>G	p.Ile353Val	1	ULD	0	-	(van der Zee *et al.*, 2017)
	9	c.1150C>T	p.Arg384Thr	2	ULD	5	2ALS	(Borghero *et al.*, 2015)
	9	c.1179A>G	p.Ile393Met	1	-	7	-	(van der Zee *et al.*, 2017)
	10	-	p.Tyr394Asp	1	-	0	ALS	(Cirulli et al. 2015)
	10	c.1190T>C	p.Ile397Thr	2	-	11	-	(van der Zee *et al.*, 2017)
	10	c.1217A>G	p.Tyr406Cys	1	-	0	-	(van der Zee *et al.*, 2017)
	10	-	p.Arg440Gln	1	SDD	1	ALS	(Cirulli et al. 2015)
	10	-	p.Cys471Tyr	1	SDD	0	ALS	(Cirulli et al. 2015)
	12	c.1424T>C	p.Ile475Thr	1	SDD	0	ALS	(Kim *et al.*, 2016)
	12	c.1426G>A	p.Glu476Lys	1	SDD	1	ALS	(Kim *et al.*, 2016)
	14	c.1544T>C	p.Ile515Thr	1	SDD	1	ALS	(Gijselinck *et al.*, 2015)
	14	-	p.Ile522Met	1	SDD	3	ALS	(Cirulli et al. 2015)
	14	c.1603G>A	p.Ala535Thr	1	SDD	10	FTD	(Gijselinck *et al.*, 2015)
	14	c.1612C>T	p.His538Tyr	1	SDD	0	-	(van der Zee *et al.*, 2017)
	15	-	p.Gln565Pro	1	SDD	0	ALS	(Cirulli et al. 2015)
	15	c.1709A>G	p.Lys570Arg	1	SDD	21	-	(van der Zee *et al.*, 2017)
	15	c.1717C>G	p.Arg573Gly	1	SDD	0	-	(van der Zee *et al.*, 2017)
	16	-	p.Gln581His	1	SDD	0	ALS	(Cirulli et al. 2015)

	19	c.1975G>C	p.Glu653Gln	1	SDD	32	-	(van der Zee *et al.*, 2017)
	20	-	p.Ile710Asn	1	SDD	0	ALS	(Cirulli et al. 2015)

Table 6.4 – TBK1 variants reported in ALS and FTD. Mutation nomenclature as recommended by the Human Genome Variation society www.hgvs.org, utilising +1 as the A of the initiator Met codon, translation start site.

Appendix C. TBK1 variants functionally characterised to date.

Type of variant	Exon	Variant[a]	Predicted aa change	Affected individuals	Affected domain	Impaired pIRF3	Impaired IRF3 binding	Impaired OPTN binding	Impaired pOPTN	Reduced NFkB activity	Splicing defect	Lower TBK1 mRNA	Lower TBK1 expression	Impaired pTBK1	Impaired TBK1 dimerisation	TDP-43 proteinopathy	Reference
Nonsense varaints	4	c.349C>T	p.Arg117X	3	KD								X			X	(Cirulli et al. 2015; Pottier et al. 2015; van der Zee et al. 2016)
	9	c.1069C>T	p.Arg357Ter	2	ULD	X		X	X				No difference	X			(Cirulli et al. 2015)
	11	c.1340+1G>A	p.Ala417X	5	SDD						X						(Freischmidt *et al.*, 2015; van der Zee *et al.*, 2017)
	11	c.1330C>T	p.Arg444X	1	SDD	X		X					X				(Tsai *et al.*, 2016)
Frameshift deletion	3	c.176dupT	p.Leu59PhefsX16	1	KD	X		X									(Pozzi *et al.*, 2017)
	4	c.358+5G>A	-	1	-						X	X	X				(Pozzi *et al.*, 2017)
	8	c. c.958delA	p. Thr320GlnfsX40	1	ULD	X		X		X							(Freischmidt *et al.*, 2015)

	10	c.1191delT	p. Ser398ProfsX11	1	-							No difference					(Gijselinck *et al.*, 2015)
	12	c.1343_1346delAATT	p.Ile450LysfsX15	3*	SDD	X		X		X							(Freischmidt *et al.*, 2015)
	12	c.1414delA	p.Ile472Serfs*8	1	SDD			X				X	X				(Kim *et al.*, 2016)
	12	c.1434_1435delTG	p.Val479GlufsX4	1	SDD	X		X		X							(Freischmidt *et al.*, 2015)
	14	c.1551_1552insTT	p.Ser518LeufsX32	1	SDD								X			X	(Gijselinck *et al.*, 2015)
In frame deletions	4	c.235_237delACA	p. Thr79del	1	KD					X			X	X		X	(van der Zee *et al.*, 2017)
	5	c.499_501delGAT	p. Asp167del	1	KD							No difference	No difference	X			(Gijselinck *et al.*, 2015)
	8	992+1G>A	p.Gly272_Thr331del	2	KD-ULD								No difference	X			ALSdb
	8	c.992+1G>T	p. Gly272_Thr331del	1	KD-ULD							No difference	X (in brain)				(Gijselinck *et al.*, 2015)
	18	c.1927_1929delGAA	p.Glu643del	6	SDD							No difference	X				(Freischmidt *et al.*, 2015; Gijselinck *et al.*, 2015)

	18	c.1928_1930delAAG	p.Glu643del	13	SDD							No difference	X	XX			(Freischmidt *et al.*, 2015; Gijselinck *et al.*, 2015)
	20	c.2138+2T>c	p.690-713del	3*	SDD	No difference	No difference	X		No difference							(Freischmidt *et al.*, 2015)
Functional missense variants	2	c.92A>G	p.Thr31Ala	1	KD								No difference	X			-
	3	c.140G>A	p.Arg47His	1	KD	X		No difference		X							(Freischmidt *et al.*, 2015)
	4	c.281T>C	p.Leu94Ser	1	KD					X							(van der Zee *et al.*, 2017)
	4	c.352G>A	p.Asp118Asn	1	KD	X		No difference									(Pozzi *et al.*, 2017)
	2	c.649G>A	p.Gly217Arg	2	KD	X		X	X				No difference	X	X		(Cirulli et al. 2015)
	6	c.687G>T	p.Arg229Ser	1	KD					X							(van der Zee *et al.*, 2017)
	7	c.731G>T	p. Gly244Val	1	KD					X							(van der Zee *et al.*, 2017)
	7	c.737T>C	p. Ile246Thr	1	KD					X							(van der Zee *et al.*, 2017)

	8	c.916C>A	p.Leu306Ile	1	KD								No difference				(Pottier *et al.*, 2015)
	8	c.923G>A	p.Arg308Gln	1	ULD	No differenec		No difference		X							(Freischmidt *et al.*, 2015)
	9	c.1070G>A	p.Arg357Gln	2	ULD	X		No difference									(Freischmidt *et al.*, 2015; Pozzi *et al.*, 2017)
	9	c.1073G>A	p.Arg358His	2	ULD								No difference	X			-
	11	c.1252A>G	p.Ile418Val	1	SDD					X							(van der Zee *et al.*, 2017)
	15	c.1676T>G	p.Met559Arg	1	SDD	X		X		X							(Freischmidt *et al.*, 2015)
	15	c.1694A>C	p.Gln565Pro	2									No difference	X			(Cirulli et al. 2015)
	20	c.2086G>A	p.Glu696Lys	3	CTD	No difference	No difference	X		No difference							(Freischmidt *et al.*, 2015; Pottier *et al.*, 2015)
	3	c.127A>G	p.Ile43Va	1	KD	No difference		No difference					No difference				(Tsai *et al.*, 2016)
	3	c.188A>G	p.Asn63Ser	1	KD					No difference							(van der Zee *et al.*, 2017)

	6	c.550A>G	p.Met184Val	1	KD					No difference							(van der Zee *et al.*, 2017)
	7	c.794T>C	p.Val265Ala	1	KD					No difference							(van der Zee *et al.*, 2017)
	8	c.881G>A	p.Gly294Asp	1	KD	No difference		No difference					No difference				(Tsai *et al.*, 2016)
	8	c.964C>T	p.His322Tyr	1	ULD					No difference							(Gijselinck *et al.*, 2015)
	9	c.1057A>G	p.Ile353Val	1	ULD					No difference							(van der Zee *et al.*, 2017)
	9	c.1179A>G	p.Ile393Met	1	-					No difference							(van der Zee *et al.*, 2017)
	10	c.1217A>G	p.Tyr406Cys	1	-					No difference							(van der Zee *et al.*, 2017)
	12	c.1412G>A	p.Cys471Tyr	1	SDD	No difference		No difference	No difference				No difference	No difference			(Cirulli et al. 2015)
	12	c.1424T>C	p.Ile475Thr	1	SDD	No difference	No difference	No difference					No difference				(Kim *et al.*, 2016)
	12	c.1426G>A	p.Glu476Lys	1	SDD	No difference	No difference	No difference									(Kim *et al.*, 2016)
	14	c.1612C>T	p.His538Tyr	1	SDD					No difference							(van der Zee *et al.*, 2017)

	15	c.1709A>G	p.Lys570Arg	1	SDD					No difference							(van der Zee *et al.*, 2017)
	15	c.1717C>G	p.Arg573Gly	1	SDD					No difference							(van der Zee *et al.*, 2017)
	19	c.1975G>C	p.Glu653Gln	1	SDD					No difference							(van der Zee *et al.*, 2017)

Table 6.5 – TBK1 variants functionally characterised in ALS and FTD. Mutation nomenclature as recommended by the Human Genome Variation society www.hgvs.org, utilising +1 as the A of the initiator Met codon, translation start site. Rows in darker grey represent variants characterised in this study.